Division of Pharmaceutical Biosciences
Faculty of Pharmacy
University of Helsinki
Finland

Biomedical applications of nanofibrillar cellulose

by

Patrick Laurén

ACADEMIC DISSERTATION

To be presented, with the permission of the Faculty of Pharmacy of the University of Helsinki, for public examination in Auditorium 2 at Infocenter Korona, (Viikinkaari 11, Helsinki), on the 29th June 2018, at 12 noon.

Helsinki 2018

Supervisors Professor Marjo Yliperttula
Division of Pharmaceutical Biosciences
Faculty of Pharmacy
University of Helsinki
Finland

Associate Professor Timo Laaksonen
Laboratory of Chemistry and Bioengineering
Tampere University of Technology
Finland

Professor Arto Urtti
Division of Pharmaceutical Biosciences
Faculty of Pharmacy
University of Helsinki
Finland

Reviewers Professor Timo Ylikomi
FICAM, Finnish Centre for Alternative Methods
Faculty of Medicine and Life Sciences
University of Tampere
Finland

Associate Professor Julien Bras
CNRS, LGP2, Laboratoire Génie des Procédés Papetiers
Graduate School of Engineering in Paper, Print Media and Biomaterials
University Grenoble Alpes
France

Opponent Professor Orlando Rojas
Department of Bioproducts and Biosystems
School of Chemical Engineering
Aalto University
Finland

ISBN 978-951-51-4350-1 (paperback)
ISBN 978-951-51-4351-8 (PDF)

Unigrafia Oy
Helsinki Finland 2018

Abstract

Hydrogels are emerging as an important source for current biomaterial design, as they often possess intrinsic physical and mechanical similarities with soft tissue, are non-toxic and biocompatible. However, many hydrogel-based biomimetic materials are either derived from limited sources, or require external activators to achieve functionality, such as chemical crosslinking or environmental cues. Furthermore, many cross-linkers used with hydrogels are toxic, and environmental cues invoke slow responses. Therefore, to function as a rational biomaterial design for a biomedical application, these properties are preferably avoided, or improved with a composite system containing two or more polymer components to overcome these limitations.

Plant-derived nanofibrillar cellulose (NFC) possesses the same intrinsic properties as many other hydrogels derived from the components of extracellular matrices (ECM). Therefore, NFC shares the biocompatibility and non-toxicity aspects of biomimetic materials. However, additional features of NFC can be exploited, such as shear-thinning properties, spontaneous self-gelation and chemical modification capabilities. Additionally, the source of NFC is practically inexhaustible, and is environmentally biodegradable, bearing no ecological burden. Therefore, when designing hydrogel-based biomaterials, NFC offers versatility, which enables the fabrication of potential biomedical applications for various purposes in an environmentally safe way.

In this thesis, a wide range of potential applications of NFC-based hydrogels were investigated. These include 3D cell culturing, *in vivo* implantation and coating systems for drug and cell delivery, controlled drug delivery and local delivery as a bioadhesive system. These methods offer insight into the versatility of NFC-based hydrogels, which could improve the future design of biomaterials, for a safer and more efficient use in biomedical applications.

Acknowledgements

This work was carried out at the Division of Pharmaceutical Biosciences, Faculty of Pharmacy, University of Helsinki. All the work put into this dissertation was made possible by the Doctoral Programme in Materials Research and Nanosciences (MATRENA), and of course the all the great minds I've had the pleasure to work with during my doctoral studies. To everyone, I would like to offer my humblest thanks, as it has been my privilege to have met you all.

I am very grateful for the guidance of my supervisors Prof. Marjo Yliperttula, Prof. Arto Urtti and Prof. Timo Laaksonen, who have supported me during my studies and research work. Prof. Urtti has always inspired me with his wisdom, be it during his lectures, personal scientific advice or the investigation of life itself, I've learnt and improved myself greatly under his guidance. Prof. Laaksonen has been an outstanding supervisor and friend, who helped me to achieve all my goals at the end (and in time!). And Prof. Yliperttula, who has been there from the beginning. Already at 2007, during my sophomore year as a pharmacy student, Prof. Yliperttula introduced me to her biopharmaceutics group. First as a research assistant and later a doctoral student, Prof. Yliperttula has always inspired me to bravely use my head for what it is there for, and face challenges head on. Always finding time for whatever it may have been, Prof. Yliperttula has been there for me, and for that I'm forever grateful.

It is my honor to have Prof. Orlando Rojas from Aalto University as my opponent, and I greatly appreciate his interest to find time from his busy schedule to attend my thesis defense. I would like to thank Prof. Timo Ylikomi from University of Tampere and Prof. Julien Bras from University Grenoble Alpes for their review and insightful comments on the doctoral dissertation manuscript. Additionally, I thank Docent Timo Myöhänen and Docent Tiina Sikanen for their participation in the doctoral thesis grading committee and attending my thesis defense.

My dear scientific colleagues and friends. I started my journey under the wings of Dr. Tatu Lajunen and Dr. Leena Kontturi, from whom I've learnt greatly about science and life. We will always have the besterestest cell encapsulation. Leena Pietilä and Timo Oksanen, without whom I would have been in deep trouble many times. Their help and support as friends and in the laboratory, has been invaluable. I thank Dr. Yan-Ru Lou for her valuable comments and input in the scientific work and friendship during office talks. I thank the whiskey club and friends: Dr. Andreas Helfenstein for being so golden, Jaakko Itkonen for being my buddy under the boat in Vantaa and being a strapping young lad, Staffan Berg for all the fish sticks and dota, Teemu Suutari for all the drunk arguments (may we never agree), Kim Ng for the best laugh, Feng Deng for anime & manga, Dr. Marinus Gerardus Casteleijn, for being the Randomest Growl (and also for nanoplotter from hell), Dr. Tapani Viitala for QCM shenanigans, Dr. Lauri Paasonen for continued interest and for being the contact man, Heikki Räikkönen for knowing everything about gadgets and technical wizardry, Dr. Fumitaka Tasaka for beer and mölkky fooooooo(!), Prof. Yuuki Takashima Miyamoto for best sensei, Kanako Niitsu for best Japanese conversation (I'm sorry), Dr. Aniket Magarkar for all the bear hugs, Dr. Sanjay Sarkhel for the philosophical discussions and best biryani in the world, Dr. Madhushree Bhattacharya for her

kindness and understanding (I know I was a tough cookie), Dr. Léo Ghemtio for making me understand modern art, Dr. Melina Malinen for the moral support, Dr. Liisa Kanninen for all the bubbliness, Nitai Peled for everything, Mikko Kunnari and Jutta Karppinen for being the best students anyone could ask, Petter Somersalo for being the voice of reason, Dr. Tiina Lipiäinen for showing how it's done, Dr. Alma Kartal-Hodzic for all the support and encouragement, Dr. Marikki Peltoniemi for all the teaching and whiskey, Otto Kari for the opinions, Dr. Dominique Richardson for the banter, Anna-Kaisa Rimpelä for the drunken banter, Riina Harjumäki for supportive discussions, Annukka Hiltunen for the cats, Mecki Schmitt for matkakalja, Dara Kien for the positivity, Tarmo Ojanen for wild west and jazz, Tomi Talja for the Grey Terminal, Chris (the useless Norwegian) and Emmi Reiersen for Cologne, Calpo, Mikko, Heikki, Jussi, Antti 1.0, Antti 2.0, Max, Kristiina, Noora, Atte and Pekka for the lohi (möh möh).

And I would like to especially thank Nicholas Andersson for being culturally superior and the Project Backfire, and Toni Linnava for being my best friend for 30 years now. Wow, 30 years already! You better appreciate it you twat. I thank the family Laurén and all my relatives for continued support over the years. And finally, Heli, who has been my closet support at work and at home. You endured everything with me, and to you belongs my deepest love and thanks. We did it together!

Contents

List of original publications

This thesis is based on the following publications:

I Bhattacharya M, Malinen M, **Laurén P**, Lou YR, Kuisma SW, Kanninen L, Lille M, Corlu A, Guguen-Guillouzo C, Ikkala O, Laukkanen A, Urtti A, Yliperttula M. Nanofibrillar cellulose hydrogel promotes three-dimensional liver cell culture. J Control Release, 164, 291-298, 2012.

II **Laurén P**, Lou YR, Raki M, Urtti A, Bergström K, Yliperttula M. Technetium-99m-labeled nanofibrillar cellulose hydrogel for *in vivo* drug release. Eur J Pharm Sci, 65, 79-88, 2014.

III **Laurén P**, Somersalo P, Pitkänen I, Lou YR, Urtti A, Partanen J, Seppälä J, Madetoja M, Laaksonen T, Mäkitie A, Yliperttula M. Nanofibrillar cellulose-alginate hydrogel coated surgical sutures as cell-carrier systems. PLoS One, 12, e0183487, 2017.

IV Paukkonen H, Kunnari M, **Laurén P**, Hakkarainen T, Auvinen VV, Oksanen T, Koivuniemi R, Yliperttula M, Laaksonen T. Nanofibrillar cellulose hydrogels and reconstructed hydrogels as matrices for controlled drug release. Int J Pharm, 532, 369-280, 2017.

V ***Laurén P,** *Paukkonen H, Lipiäinen T, Dong Y, Oksanen T, Räikkönen H, Ehlers H, Laaksonen P, Yliperttula M, Laaksonen T. Pectin and mucin enhance the bioadhesion of drug loaded nanofibrillated cellulose films. Pharm Res, 35, 145, 2018. *Equal contribution.

The publications are referred to in the text by their roman numerals (I-V). Reprinted with the permission of the publishers.

Author's contribution

Publication I

The author designed and performed the experiments for hydrogel injectability and diffusion/permeation studies. The author participated in biomaterial preparation, cell culturing and viability studies with other co-authors. Additionally, the author was involved in the analyzing of rheological data and wrote short drafts concerning hydrogel injectability and permeability for the manuscript.

Publication II

The author designed the experiments with the supervisors and co-authors. The author prepared and performed all radiolabeling procedures and stability tests. The author performed all the small animal studies under the supervision of PhD Mari Raki and analyzed all the data from SPECT/CT. Pharmacokinetic simulations were done by the author under the supervision of professor Arto Urtti. The author was responsible for all the coordination needed to successfully carry out the research, writing the manuscript and submitting the final paper after the comments from the co-authors.

Publication III

The author designed the experiments with the help of Petter Somersalo and Mari Madetoja. The author performed all rheological studies and data analysis. The author and Petter Somersalo performed the biomaterial preparation, confocal microscopy and cell studies. The author participated in the *ex vivo* suturing performance studies with Mari Madetoja. The author wrote the manuscript and prepared the final paper after the co-author comments. The author was responsible for submitting the final paper.

Publication IV

The author designed and performed the rheological, drug release and solid state analysis experiments with the supervisors and co-authors. The author participated in scanning electron microscopy with Heli Paukkonen and professor Timo Laaksonen. The author actively participated in the writing of the manuscript and revising the final paper after co-author comments with Heli Paukkonen.

Publication V

The author designed the experiments in equal responsibility with Heli Paukkonen with the help of supervisors and co-authors. The author participated in all experiments and analyzed the results together with Heli Paukkonen. The author was responsible for writing the manuscript together with Heli Paukkonen and after co-author comments revised the final paper.

Abbreviations

16HBE	Human bronchial epithelial cell-line
ANFC	Anionic carboxylated nanofibrillar cellulose
ARPE-19	Spontaneously arising human retinal pigment epithelium cell-line
BAC	Bacterial artificial chromosome
BALB/c	Bagg albino (mouse strain)
BC	Bacterial cellulose
BLT	Bone marrow, liver, thymus
BMECs	Brain microvascular endothelial cell-line
BSA	Bovine serum albumin
CD34+	Human hematopoietic stem cell-line
CMC	Carboxymethylcellulose
CT	X-ray computed tomography
ECM	Extracellular matrix
FD	Freeze-dried aerogel
GM-CDF	Granulocyte-macrophage colony-stimulating factor
GI	Gastro-intestinal
HA/CS	Hyaluronic acid-chitosan
HCEC	Human corneal epithelial cell-line
HepG2	Human hepatocellular carcinoma cell-line
Hu-PBL	Human peripheral blood lymphocyte
Hu-SCR	Human SCID repopulating cell
HSA	Human serum albumin
^{123}I- β-CIT	Iodine-123-(2-beta-carbomethoxy-3-beta-(4-iodophenyl)-tropane)
IL-n	Interleukin-n (n indicates the specific type of interleukin)
IL2rg^{null}	Interleukin-2 receptor gamma chain
kDa	Kilodalton (also refers to an efficiency value in DotA2)
KETO	Ketoprofen

LZ	Lysozyme
M-CSF	Macrophage colony-stimulating factor
MCC	Microcrystalline cellulose
MRI	Magnetic resonance imaging
MZ	Metronidazole
NAD	Nadolol
NFC	Nanofibrillar cellulose
NFCA	Nanofibrillar cellulose-alginate
NOD	Non-obese diabetic
PDEAAm	Poly(N,N-diethylacrylamide)
PDMS	Polydimethylsiloxane
PDX	Humanized patient-derived xenograft
PEG	Poly(ethylene glycol)
PNIPAAm	Poly(N-isopropylacrylamide)
PRKDC	Protein kinase DNA-activated catalytic polypeptide
PVA	Polyvinyl alcohol
PVAc	Polyvinyl acetate
PVPA-co-AA	Poly(vinylphosphonic acid-co-acrylicacid)
RGD	Arginylglycylaspartic acid
RS/P	Resistant starch/pectin
SCID	Severe combined immunodeficiency
SEM	Scanning electron microscopy
SPECT	Single-photon emission computed tomography
SIRPα	Human signal regulatory protein alpha
SK-HEP-1	Human adenocarcinoma-derived endothelial cell-line
TPO	Thrombopoietin
TR146	Human squamous cell carcinoma cell-line
YM	Young's modulus

1 Introduction

A biomaterial can be classified basically as any material that interacts or is in contact with living surroundings, such as tissue or cells. The use of biomaterials date all the way back to the ancient Egypt, where archeological findings have revealed possibly the first prosthesis with functional properties [1]. An anatomically correct wooden big toe was crafted and implemented with surgical expertise to allow the patient to move normally. The added function of the biomaterial construct would therefore allow it to be classified as the first biomedical device. Biomedical devices take advantage of engineering sciences to fabricate and deploy biomaterials for healthcare purposes. This leads to the field of biomedical engineering, where engineering and medicine are considered as the two sides of the same coin. It is an interdisciplinary field, where biomedical devices are designed and utilized in therapeutics, diagnostics and tissue engineering. However, we are no longer bound to metal, wood and leather, and new biomaterials and their applications emerge constantly, with personalized functionality and structural design that also resembles the original human physiology [2].

It can be rationalized that to reach the optimal biomedical device functionality, it would be preferable to imitate the original tissue function as closely as possible, which the device is designed to repair or replace. However, different tissues have different characteristics. Therefore, there is no universal option to solve the problem. Additionally, the extracellular matrix (ECM) is dynamic, and is affected by various factors at various times [3]. And as such, the literature on various biomaterials and their usage is vast; therefore, this thesis focuses mainly on three themes, which can be considered as the core elements of the literature review part. The key aspects are the current technologies and methods, such as humanized animal models and microfluidics, that are used to mimic human physiology and to study the biological processes and disease states. This is important for understanding the underlying phenomena in designing the biomedical devices and their applications. The biomaterial designs used in these applications need to resemble the microenvironment they are targeted at. The focus is in safe and efficient treatments, such as limiting host responses and achieving physiological functionality. The main discussion follows the biomaterial design based on hydrogels, as they are one of the main source of novel biomedical devices [4,5]. As this thesis is specifically aimed at exploring the use of hydrogels based on plant-derived native nanofibrillar cellulose (NFC) and anionic carboxylated nanofibrillar cellulose (ANFC), the final theme is focused on the literature of these biopolymers in the biomedical field.

Cellulose-based materials, NFC and ANFC are generally considered as safe, biocompatible and non-toxic biomaterials, with low cost and a renewable source [6]. The architecture of cellulose follows a hierarchical path from the plant tissue all the way to individual cellulose molecules. NFC is usually derived from plant ECM, where the individual crystalline cellulose microfibrils are isolated from the raw material. Single microfibrils (i.e. nanofibrils) are typically 3-4 nm in diameter and consists approximately 30-40 cellulose polysaccharide chains [7]. Therefore, NFC

is a finely structured hydrogel composed of single isolated cellulose microfibrils. And because of the hydrogel nature, NFC has a very high content of water, which is bound within the matrix by strong hydrogen bonding to the hydroxyl groups present in the cellulose molecules. Therefore, NFC has some intrinsic resemblance to soft tissue mechanically, which enables cell culturing in a natural 3D *in vivo*-like environment [8,9]. Additionally, NFC has extensive chemical modification capabilities, due to the available functional hydroxyl groups. These properties make NFC an excellent candidate for potential hydrogel-based biomedical device applications.

The experimental section of this thesis considers, evaluates and characterizes different types of potential pharmaceutical and biomedical applications for NFC-based hydrogels, such as 3D organoid development, injectable implant, surgical suture coating, controlled drug release application and bioadhesive film formulation. The main aspect focuses on the use of NFC and ANFC by themselves or as a combination of functional natural materials, such as alginate, pectin and mucin, without the need for toxic cross-linkers. Materials and their combinations are characterized by their rheological properties and *in vitro*/*in vivo* suitability, with some consideration about their mechanical properties. Drug release and cell carrier properties are investigated in addition to surface interactions, such as cell attachment, cell encapsulation and bioadhesion.

The aim was to develop rational, safe and efficient applications for the purpose of improved healthcare. The advantages and limitations of the NFC-based applications are discussed and compared with other studies available in literature in their respective experimental sections. These applications could potentially improve the therapeutic delivery of drugs and cells to their target locations, which take advantages of NFC characteristics and established treatment methods, such as surgical suturing, minimal-invasive and non-invasive administration. Therefore, it is my opinion that the combination of established methodology and the use of NFC-based materials would prove useful in designing new biomaterials for various biomedical application purposes.

2 Mimicking the *in vivo* environment

In vitro methods have a long history in biomedical research, and have been widely developed and utilized to enable isolated experimental systems, such as cell and tissue cultures [10]. These are designed to function as convenient and simple representations of physiological aspects under study; however, the correlation between the *in vitro* experiment and the physiological response of a fully functional and complex organism remains a challenge [11]. The differences between *in vivo* and *in vitro* environment have therefore been acknowledged, and new methods have been investigated to bridge the gap in between [12]. These differences can be categorized in some key elements (Figure 1), which represent various cues in the extracellular microenvironment that regulate cell behavior, such as cell differentiation, proliferation, migration, adhesion and polarity [13]. These elements may also change with time, which generates a dynamic environment in contrast to the static conditions often present in *in vitro* methods.

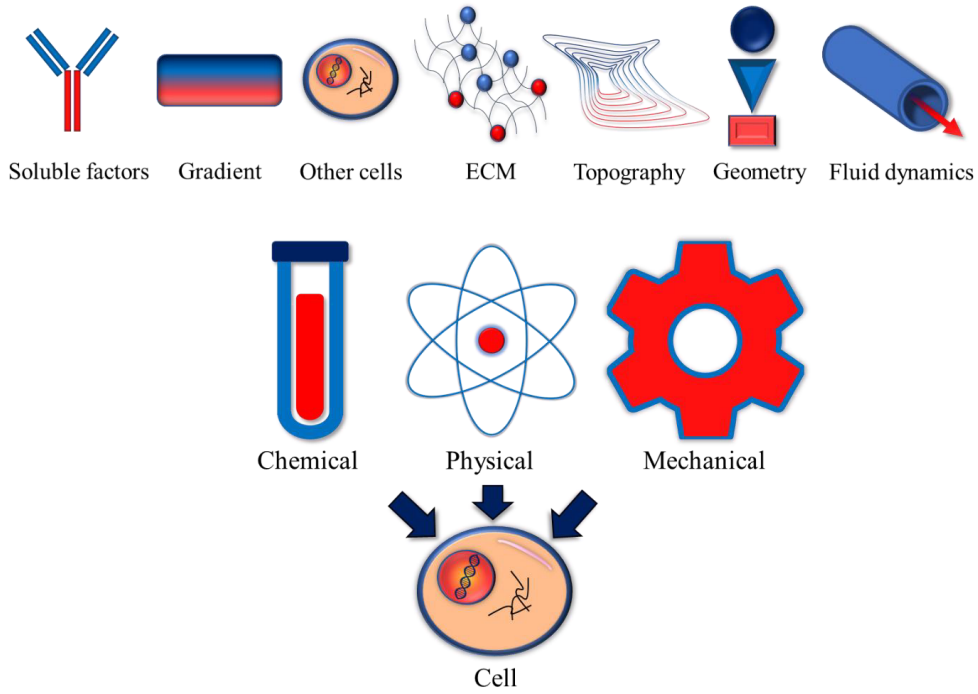

Figure 1. *Key factors affecting cell behavior. Chemical, physical (static) and mechanical (dynamic) cues in the microenvironment trigger various cellular responses.*

The key elements affecting cell behavior are either chemical, physical or mechanical [3,14,15]. Biochemical and physical cues also provide a gradient, which in turn can be linear or non-linear [16]. Examples of chemical gradients are oxygen, hormones or other soluble factors, which can affect cell behavior. Physical and mechanical cues relate to the extracellular surroundings, which the cell can sense and interact through integrin mediated signal transduction [17]. Examples of physical cues are surface topology [18], density [19] and porosity [20], and for mechanical cues, hardness/softness [21] and stiffness [22]. Additionally, tissue functions can trigger mechanical cues, for example tissue deformation (e.g. the compressive forces of the gut) [23] or the shear forces of the blood flow [24,25]. These cues provide a complex system for guiding cell behavior, such as migration, adhesion, proliferation, apoptosis and differentiation.

Many elements in the microenvironment can interact in numerous ways, for example cell-cell or cell-ECM interactions can be both chemical and physical [26-30]. All these various cues and interactions contribute to the development [31] and maintenance of a complex system (i.e. homeostasis) [32]. Additionally, these cues need to be controlled spatially and temporally to achieve better correlation with physiological responses. Currently, *in vitro* methods cannot reproduce the complexity of an intact organism, and therefore the output of the method is always limited due to the lack of physiological characteristics. This sets an imposing challenge, not only to understand all the underlying physiological mechanisms and the dynamic interactions within the whole system, but also to reproduce them. Fortunately, there is an increasing interest in pursuing these challenges and recently many attempts have been made to improve the correlation between *in vitro* and *in vivo* [33-38]. For example, several microfluidic systems have been developed to help overcome some of these challenges [39-41].

Microfluidics

Microfluidics combined with microchip technologies enable the control over many elements necessary to mimic physiological environments [42]. These systems are often termed as “human-on-a-chip”, or “organs-on-a-chip”. These devices allow continuous, real-time analysis and imaging of cell cultures. Typically, most microfluidic devices are fabricated with the soft lithography method, which is an extension of photolithography [43]. First a photo-sensitive material (a.k.a. photoresist), such as resin, is deposited on a silicon wafer (i.e. the substrate). The thickness of the resin layer determines the sizes of the micro-channels, which usually range from a few hundred nanometers to tens of micrometers. On top of the resin is placed a protective layer, a photomask, which includes the micro-channel pattern etched on the mask. The photomask is then exposed to UV light, which cures the resin at the unprotected areas of the photomask, i.e. the micro-channel pattern. The protective mask is removed and the uncured resin is dissolved leaving a master mold, which can be further used in soft lithography. Soft lithography utilizes a wide variety of elastomeric materials, most commonly polydimethylsiloxane (PDMS). Advantages of PDMS are biocompatibility, transparency, ease of use and low cost. The molding process begins with PDMS solution deposited on top of the master mold acquired by photolithography. PDMS is cross-linked, for example with a

hydrosilylation reaction [44], to harden the solution into a PDMS block. Once the block is obtained, it can be further modified with tube connectors, such as inlet and outlet drilling or punching. The device is completed when the block is placed typically on top of a glass slide and closed off with plasma bonding. Afterwards the device can be connected to pumps and reservoirs, or combined with other microfluidic devices.

Microfluidic cell culture devices provide temporal control in the system, including a chemical gradient with mechanical forces, such as fluidic shear and compression [43]. Specific responses can be analyzed in single cell- and co-cultures, in addition to recreating interfacial processes such as the blood-brain-barrier [45]. The model was capable of mimicking blood-brain-barrier physiology with frequent flow fluctuation for up to 10 days with well-organized tight junctions and morphology of brain microvascular endothelial cells (BMECs) already on day 3. Therefore, an important aspect in mimicking physiological conditions is precise flow control. Flow control can be achieved through switching between efficient mixing turbulent flows and minimal mixing laminar flows in a series of channels to create gradients. Fluid-to-cell ratios can be designed to match physiological conditions to mimic physiologically relevant extracellular environments, which assist in tissue development. For example, C2C12 myoblasts were induced into different states of cell differentiation with a microfluidic system [46]. Microfluidic devices can also be designed to function autonomously, or as self-regulating systems [47]. For example, it was shown that the cell protein concentration could be precisely controlled with the feedback-loop parameters regulating the loop response dynamics [48], which are one of the most important mechanisms in maintaining homeostasis.

Microfluidics offers a variety of means to improve therapeutic strategies with artificially recreated biological complexes. Therefore, the biomaterial design of hydrogels can be also improved with advanced microfluidic systems modeling the *in vivo* environment. A single cell capture system was shown to be effective in detecting circulating tumor cells [49]. The system was designed to restrict cell flow in a mixed population to hold and trap cells of interest selectively. A strong correlation was found between the device and tumor biopsies, indicating a physiologically relevant sampling and diagnostics method. Although the device did not mimic a specific organ or organs to create the artificial environment, however, such devices have been investigated [50]. A multi-organ system was designed to mimic metastasis formation in a lung cancer model. Human bronchial epithelial (16HBE) cells were cultured at the primary site, and three separate downstream cultures consisted of astrocytes, osteoblasts and hepatocytes. Lung cancer cells were introduced with the 16HBE cells and allowed to grow at the primary site. The invasion to downstream sites (brain, bone and liver) was then investigated. The metastasis progression was effectively recreated and analyzed in a system, which mimicked the physiological characteristics of multiple living organs. Stem cell research has also benefited from microfluidics. Stem cell differentiation responses can be detected with graded concentration ranges of growth factors [51]. Advantages of such devices are the use of single culture systems, which enable the precise optimization of stem cell use in tissue engineering and transplantation. Recently, stem cell applications have been combined with functional hydrogels [52]. Precisely sculpted or designed 3D structures enable biochemically and

physically relevant environments, i.e. microgels, which can be further tailored with microfluidics, especially with the use of stem cells that are known to be highly sensitive to their surroundings (Figure 2). Functional microgels and -fluidics combined can provide efficient and precise tools to construct complex biological settings to mimic *in vivo* microenvironments, which could aid in the design of more advanced biomaterials and biomedical devices.

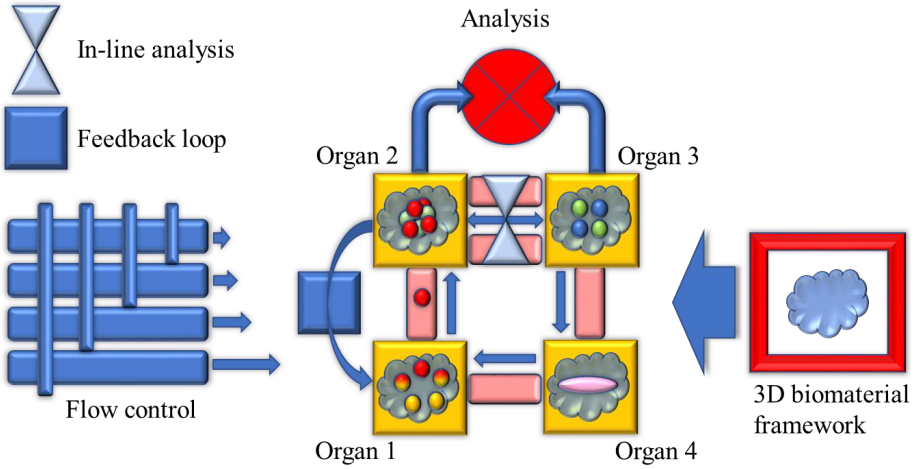

Figure 2. *A simplified schematic of a microfluidic "organs-on-a-chip". The chip contains multiple organs represented by their respective chambers. Each chamber can house cells in various states of differentiation. The cells can be seeded individually, as co-cultures or patterned in multiple ways. Microgels can be implemented with various properties to mimic different organ characteristics. Fluid flow can be adjusted in individual channels with a flow control system, which also controls the peripheral cell-signaling gradient between the chambers (e.g. a feedback loop). And finally, analysis can be made in-line or individually from the outlet of each chamber. For example, such designs can potentially improve the understanding of the mechanisms in disease onset and pathogenesis. Organs-on-a-chip microfluidic systems could potentially advance the designing of biomedical devices for the treatment of various diseases.*

Humanized animal models

Despite the advantages of microfluidics and stem cell research, using cell or tissue samples from human donors is not without its problems, such as ethical and logistical issues. Humanized animal models can provide alternative ways to achieve physiologically relevant information [53], which, as was discussed with microfluidics, also assist in the development of hydrogel biomaterials for biomedical purposes. The development of humanized mice has occurred in three consequent breakthroughs, beginning with the inactivation of the immune system of mice [54]: (i) the discovery of severe immunodeficiency (*scid*), facilitated by a PRKDC (protein

kinase DNA-activated catalytic polypeptide) gene mutation in CB17 mice, which enabled host mice to be relatively receptive towards heterologous cell transplantations; (ii) the development of non-obese diabetic NOD-*scid* mice, allowing higher levels of engraftment with the decrease of host NK-cell activity; (iii) targeted mutations at interleukin-2 receptor gamma chain ($IL2rg^{null}$), which disabled several interleukin cytokine receptor activations, resulting in a lack of adaptive immunity and absence of NK-cells. The most commonly used strains of mice concerning the breakthroughs above are NSG, NOG and BRG [55]. These strains have been shown to model physiological responses more accurately than any earlier strain.

The incorporation of the human immune system in the animal is then enabled, as the strain (host) immune system has been severely compromised. There are three typical models of humanization [55]. Human peripheral leukocytes are injected into the host Hu-PBL-SCID model. The method is excellent for T cell function investigation, but with a drawback of relatively short experimental window. In the Hu-SCR-SCID model, human CD34+ hematopoietic stem cells are administered intravenously. This provides the model with a complete human immune system; however, bone marrow generated cells are detected at lower levels. The third model, aptly nicknamed as the "BLT" (bone marrow, liver and thymus), utilizes human fetal liver and thymus transplants at the host kidney with an injection of human CD34+ hematopoietic stem cells. A fully functional human immune system is developed (including a mucosal immune system), but this model also suffers from a restricted experimental window.

Specific models are therefore required depending on the study needs. However, numerous studies have already been performed with the above-mentioned strain and model combinations. For example, a major field of investigation is infectious diseases such as HIV, in which the BLT model especially has proven valuable due to the functioning human mucosa enabling the use of both vaginal and rectal transmission routes [56]. Early viral infection dynamics can be investigated, which allows the evaluation of therapeutic prevention in addition to treatment and potential eradication. Another example is *Plasmodium falciparum* (malaria) where the full life cycle of the parasite could be replicated [57]. This enables the study of human infected species in animals with specific responses to treatments that reflect human physiology. Incidentally, the investigation of infectious diseases has also led to new discoveries. In a study of NSG Hu-SRC-SCID infected with influenza (H1N1), it was observed that the monocyte and macrophage development was defective, but could be stimulated with the administration of a cytokine macrophage colony-stimulating factor (M-CSF), which led to decreased viral transcription factors and increased inflammatory cytokine levels [58].

Liver is the most important organ in human metabolism, including the metabolism of drug compounds. The repopulation of host hepatocytes with human cells enables the study of drug metabolism, toxicity and interactions in humanized mice [59,60]. A chimeric mouse model was developed, where human specific gene expression of several CYPs were observed [61]. 1A1, 1A2, 2C9, 2D6 and 3A4 were identified to match the donor values. Additionally, the metabolic function of the model was evaluated with all the major human CYP enzymes present, including

2C9 [62]. Drug compounds with known specific metabolic activities were measured with microsomes isolated from the chimeric mice. High level of human transplantation was observed by monitoring the levels of circulating human albumin. However, high levels of secretory complement factors from the transplanted human cells resulted in proteolytic toxicity against host organs in both studies [61,62]. Therefore, the model suffers from a relative short life span of the mice. Despite the apparent issues, these models can metabolize drug compounds on comparable levels with the human liver. Additional improvements have been made with human hepatic stem cells to ensure 100 % rate repopulation, which functioned as the actual human liver would in metabolizing ketoprofen and debrisoquine [63]. The humanized liver models also enable the study of drug compounds, which are inherently toxic to mice, such as furosemide [64], which renders them near unusable in standard small animal studies. The differences in mouse and human metabolisms are therefore reduced and similar metabolic characteristics can be observed, increasing the translational power between human and humanized animals.

Another field that has been extensively taken advantage of humanized animals is cancer research. The ability to investigate the interactions between tumors and human immune system in animal models has given valuable information in tumor immune escape and the effectiveness of antibody treatment and immune modulation [55]. For example, a NSG Hu-SRC-SCID model was developed for studying immune modulation in cancer treatment and resistance of human breast cancer [65]. A fully developed human immune system was observed, with the maturation and activation of T-cells in the presence of the tumor. Interleukin-15 (IL-15) treatment induced NK-cell activation, indicating potential immune modulation therapeutic possibilities. However, many current models have limitations concerning cancer research, such as inadequate tumor heterogeneity [66], or the lack of proper cytokine signaling factors [67]. As discussed earlier, the innate immune cell development is defective without cytokine enhancement (i.e. only low levels of macrophages, monocytes and NK-cells are present) [68]. To overcome this issue, a humanized mouse model called MISTRG Hu-SRC-SCID, was developed [69]. The human genes encoding the cytokines interleukin-3 (IL-3), M-CSF, granulocyte-macrophage colony-stimulating factor (GM-CDF) and thrombopoietin (TPO) were knocked in to their respective loci. Additionally, a bacterial artificial chromosome (BAC) transgene was utilized to encode the human signal regulatory protein alpha (SIRPα) responsible for integrin associated protein (CD47) interactions. With the proper signaling mechanics functioning, high and diverse levels of innate human immune cells were observed. Humanized patient-derived xenografts (PDX) have been suggested to improve tumor heterogeneity [55,66]. The PDX model is an advancement of human cell-line derived humanized models as the original complex tumor microenvironment can be retained. Despite the ability to recapitulate the complete tumor microenvironment, the PDX is limited, as maintaining a fully functional human immune system in the host that matches the patient is still a challenge [66].

Humanized animal models have also been used in studying allograft rejection and autoimmunity [70,71]. As described earlier, the humanized immune system provides an excellent platform for physiologically relevant observations in mice. This has also lead to

numerous studies investigating the mechanisms allograft rejection in animal models [70], which could provide to be useful in developing treatments and methods to prevent transplant rejection. Another example of functional humanized animal model use is the study of underlying mechanisms of type 1 diabetes, an autoimmune disease [71]. These models could provide insight of the mechanism and onset of the diseases, which would further improve in the development of therapies to combat autoimmunity. Therefore, humanized animal models are able to mimic the human *in vivo* environment closely, and are powerful tools to understand many mechanisms and processes of human development and diseases, but also provide methods to study interactions, such as pharmacodynamics and pharmacokinetics. The understanding of these aspects is also essential in designing functional biomaterials.

3 The design of hydrogel biomaterials

While microfluidics and humanized animal models are excellent tools, an alternative approach is to engineer ECM-mimicking hydrogels. Additionally, both systems could benefit from carefully designed hydrogel biomaterials, such that they bring structure and function into the system [72], or act as cell carriers [73]. However, the ECM is a very intricate and complex environment with multiple functions in cell interactions and regulation [74]. The ECM is responsible for the structural surroundings and various cues, which are mainly composed and mediated by collagens, elastins, fibronectins, laminins, proteoglycans and other components totaling approximately 300 different proteins (i.e. the matrisome) [75]. Every cell type in the human body has a reciprocal effect with the ECM, this interaction can take place either directly or indirectly (Figure 3), for example as a direct cell anchoring platform via the interaction of actin components in the cytoskeleton or as an indirect interaction via the reservoir of various growth factors [76]. The ECM is in a constant cycle of being made by secretory components, remodeled and degraded by the cells, depending on the cell type and feedback loops. The dynamic interaction therefore defines the cell microenvironment and regulates cell behavior. However, it is not necessary to fully mimic the microenvironment and its every single detail, as there are design parameters that can be "tuned". For example, with hydrogels the mechanical properties can be adjusted to correlate with the surrounding tissue [77], chemical modifications can guide cell behavior [78] and rheological characteristics can be used to affect solute diffusivity [79]. These design aspects are discussed in more detail in the following paragraphs.

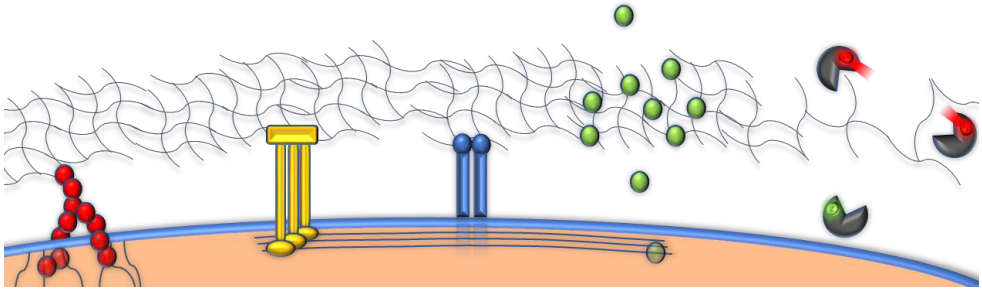

Figure 3. *Types of cell-ECM interactions in regulating cell behavior. The signaling mechanisms of ECM-cytoskeleton anchoring (red), trans-membrane- (yellow) and membrane-bound (blue) signal transduction and the biochemical gradient (green) guide cell behavior through mechanical and biochemical changes in the microenvironment. ECM can also be regulated and remodeled by the cell, by secreting new components (not shown) and through enzymatic degradation (black).*

Hydrogels are hydrophilic polymer structures that are capable of retaining large amounts of water similarly to soft tissue, while remaining structurally stabile [80]. The bound water within the polymer network enables the permeation and diffusion of nutrients and secreted molecules. The three-dimensional network is formed by entangled insoluble polymer chains with tunable properties, through which it is possible to affect the hydrogels mechanical properties (i.e. rheology), porosity, biodegradability and biocompatibility. Forces responsible for holding the hydrogel structure intact are often based on ionic crosslinking, hydrogen bonding, polymer entanglement and hydrophobic effects [81]. Hydrogels that react to external stimuli are loosely called "smart" hydrogels [82,83]. In these, external stimuli can produce changes in the network, which in turn can modify various properties of the hydrogel.

Environmentally sensitive hydrogels

A common form of environment sensitivity in hydrogels is temperature sensitivity [84]. Temperature sensitivity is often induced by propyl, methyl and ethyl groups present in the polymer chain network. Two widely used temperature-responsive polymers are poly(N-isopropylacrylamide) (PNIPAAm) and Poly(N,N-diethylacrylamide) (PDEAAm). For example, carbon nanotube reinforced PNIPAAm hydrogel was used with cardiac stem cells as an injectable transplant in a rat myocardial infarct model [85]. The *in-situ* implantation was enabled by temperature-sensitive gelation of PNIPAAm at above 32°C. Another example is the use of PDEAAm to enable temperature controlled molecular recognition [86]. It was observed that ligand binding sites could be blocked and unblocked by exploiting the phase transition behavior of streptavidin conjugated PDEAAm. At temperatures lower than 20°C, the polymer extends and acts as a steric hindrance, therefore preventing the binding of large biotinylated proteins. At temperatures higher than 30°C, PDEAAm begins to collapse, enabling the binding of large proteins as the steric hindrance is reduced at the binding site.

Environmental pH can also be utilized to induce changes in hydrogel polymer structure. Serum albumin was used to prepare autologous hydrogels with an oxidative reduction reaction [87]. The albumin hydrogel gelation occurs at near physiological pH (6-8), while its rheological properties, swelling and rate of biodegradation, could be controlled by modifying the albumin concentration. Albumin hydrogels are biocompatible and biodegradable, and additionally they can be fabricated from a material that is derived from the patient's own serum. Naturally, physiological pH and temperature are important features that can be exploited in hydrogel and biomaterial design. However, additional environmental cues have been investigated in inducing hydrogel reactions.

Hydrogels can be designed to respond to various other external triggers, such as electrical fields [88], magnetic fields [89] and light [90]. In these studies, the hydrogels were investigated and evaluated in drug delivery and stem cell release/recovery applications. It is important that responses can be induced by external sources outside the human body. One great example is the eye, where the distinct physiological features allow the use of an external source (e.g. light). Environmentally sensitive hydrogels in ophthalmic therapy benefit from the availability of this external trigger, as it reduces invasiveness, enables temporal control and increases target selectivity [91]. Environmental triggers can also provide measures for *in situ* formation of implants with the use of injectable hydrogels [92]. However, hydrogels prepared with chemical cross-linking can exhibit cytotoxicity, as the reaction residues and initiators can remain within the hydrogel without thorough and complete purification processes.

Synthetic and bio-based polymer hydrogels

Biocompatibility is a clear challenge in hydrogel design, as there is a paradox of contradictory properties which are both desirable. The paradox arises from the need for the material to be inert and non-inert at the same time. A hydrogel needs to be inert in a way that it does not invoke host responses against itself, but also must regulate host responses in a distinct and rational manner to achieve its full potential (i.e. is bioactive). Many natural, or bio-based, hydrogels, such as polysaccharides and polypeptides, are intrinsically physiological, as they are derived from or are actual components of *in vivo* ECM [93]. Collagen is the most abundant protein in mammals and has been widely used in biomedical research [94,95]. Collagen supports cell adhesion, is biodegradable *in vivo* and can be used as an autologous material [96]. For example, dopaminergic neuron survivability was significantly increased in a rat model of Parkinson's disease [97]. Dopaminergic neural rat progenitor cells were encapsulated within a glial-derived neurotrophic factor loaded collagen hydrogel. The combination of the trophic factor and collagen encapsulation resulted in an increased cell efficiency and enhanced motor function. Further examples of animal-based hydrogels are fibrin, hyaluronic acid and chitosan. Fibrin hydrogels have been widely used as injectable cell carrier scaffolds [98]. Fibrin hydrogels are prepared from pooled from plasma, and as such, much like collagen, they can be used as autologous materials. Additionally, fibrin self-assembles *in situ* and therefore possesses environmentally responsive properties.

Hyaluronic acid and chitosan hydrogels and their derivatives have been used and studied individually in biomedical applications for decades [99,100]. However, with the emerging "next generation" hydrogels, the tunable properties can be enhanced with co- and multi-polymer design, such as hyaluronic acid-chitosan (HA/CS)-hydrogel [101]. HA/CS hydrogels encapsulated with fibroblasts were prepared for the treatment of abdominal wall defects. The hydrogel design was investigated to mimic abdominal wall tissue microenvironment. It was observed that the cytokine levels with the HA/CS hydrogel correlated with *in vivo* tissue in wound healing, which was a result of macrophage phenotype modulation. With the use of HA/CS hydrogel in an abdominal defect rat model, the trophic factors were significantly up-regulated and fibroblast/macrophage recruitment was enhanced resulting in promoting host ECM regeneration and wound healing. Further applications that could promote clinical uses of co-polymeric hydrogel design, is the ability to functionalize existing treatment methods, such as surgical operations [102]. This aspect is discussed in more detail in the experimental part of this thesis in a functional hydrogel *ex vivo* suturing performance study **(III)**. Matrigel™ is a commercialized "next generation" hydrogel reconstituted originally from mice Englebreth-Holm-Swarm tumors [103]. It naturally mimics the basement membrane matrix with an assortment of ECM components, such as laminin and collagen, and also includes various growth factors and proteases. Therefore, it has been widely used for example as a coating for cell cultures platforms [104-106], as a reconstituted basement membrane it provides many ECM-like attributes. Recently, a natural 3D hepatic structure was fabricated with PDMS molds using a viscoelastic lithography method and matrigel-collagen hydrogel mixtures [107]. The rat primary hepatocyte and human umbilical vein endothelial cell co-cultures provided improved hepatic function in a finely controllable microstructure matrix, which could be utilized in studies requiring well defined soft tissue like microenvironments.

Despite the excellent biocompatibility of autologous materials, such as collagen, fibrin and hyaluronic acid, these materials are available from a very limited source (i.e. the patient him-/herself). Additionally, homologous materials or allografts (from donors of the same species) carry the risk of transmittable infection agents. Therefore, materials derived from more abundant sources are desired. Such materials as alginate, lignin or NFC, have been found to be biocompatible in various implantation applications and could be useful as alternative biomaterials [108-110]. However, while biocompatibility is a critical property to have, various additional features define these materials and their potential use as a biomaterial in biomedical applications.

Alginate is an anionic biopolymer generally derived from seaweed. It has been extensively studied as an injectable hydrogel for various biomedical purposes, such as cell delivery [111]. Alginate has been demonstrated to have tunable properties. For example, disodium phosphate (Na_2HPO_4) mediated the modulation of physical and mechanical properties of an alginate hydrogel [112]. The ability to tune the mechanical properties enables the preparation of hydrogels that match their respective characteristics in various tissues. This is an important issue, as there are multiple elements in organ function, which are affected by the mechanical properties of the ECM [113]. Stiffness, for example, has an important role in cell differentiation

and organ morphogenesis. In next generation hydrogels, alginate has been used in a multi-polymeric hydrogel with gelatin and collagen [114]. The system was designed to mimic corneal ECM, where bio-printing was utilized to form specific 3D hydrogel structures. The structures were prepared with human corneal epithelial cells (HCEC) encapsulated within the bio-printing ink (i.e. the alginate-gelatin-collagen hydrogel), which resulted in faster cell proliferation and improved the expression of HCEC specific protein markers. With the ability to fabricate finely defined structures with tunable mechanical properties, careful hydrogel designs therefore enable systems that are able to closely mimic the *in vivo* environment. For this apparent reason, many studies today have focused on finding new materials to further the biomaterials research.

Other plant-derived hydrogels, such as lignin-derived hydrogels and NFC hydrogels, have been investigated in biomedical research. Lignin-based hydrogels are typically isolated and produced from wood pulp (called the Kraft process) and co-polymerized, for example with cellulose, or obtained by chemical cross-linking [115]. However, a newly discovered method also enables the biomodification of Kraft processed lignin to synthesize insoluble lignin-based hydrogels without chemical cross-linking [116]. With the use of fungi, intermediate lignin polymer hydrogels were produced. Afterwards, the intermediate hydrogels were washed with various solvents to acquire insoluble lignin hydrogels with different, solvent dependent properties, such as swelling and stability of the hydrogel. Another example of lignin-based hydrogels is lignin-cellulose beads with immobilized lipase [117]. It has been suggested that these beads could be utilized in biomedical studies with biocatalytic functions provided by the immobilized enzymes.

Nanofibrillar cellulose is a polysaccharide typically isolated from wood pulp or produced with bacterial fermentation [118]. Mechanical shearing and enzymatic treatment methods result in several micrometers long cellulose fibrils and fibril aggregates typically 3-20 nm thick [119,120].The rheological properties of NFC hydrogels are dependent on the fibril concentration. However, the storage modulus is relatively frequency independent, which indicates that NFC retains its viscoelastic properties even at very low concentrations. This could be beneficial in adjusting the mechanical properties to correlate with different tissue types or to regulate cell behavior. For example, NFC has been shown to promote cell polarization and differentiation in hepatic progenitor cells [121]. This study is discussed in more detail in the experimental section of this thesis **(I)**. Additionally, human embryonic stem cell pluripotency could be maintained in NFC cultures [122]. This culture system mimics the microenvironment of the anatomical stem cell niche, which is the biological location for stem cell regulation. Therefore, enabling further utility and flexibility of the stem cell culture, as it can be transformed between 3D and 2D, without affecting the *in vivo*-like cell organization, while the differentiation behavior of the stem cells could be adjusted at any step during the cell culture.

However, as mentioned earlier, purely natural hydrogel properties are often controlled by adjusting the polymer/fiber concentration. This also causes changes in the hydrogel network properties, such as porosity or active site/scaffold fiber density [123]. Additionally, the source has a defining effect to the product, especially with hydrogels such as Matrigel™, where the

source variability between different production lots has been shown [124]. Therefore, synthetic materials could provide means to improve on these issues [123]. Poly(ethylene glycol) (PEG)-hydrogels are widely used synthetic hydrogels, with properties useful for the hydrogel design, such as controllable mechanical properties and tunable matrix network architecture and chemical compositions. However, PEG itself is biochemically inert [125]. Therefore, modifications have been investigated to improve PEG usage in biomedical and pharmaceutical research. For example, PEG hydrogels were prepared by incorporating synthetic protein grafts in the matrix structures to induce cell adhesion [126]. In this study, photopolymerization was used to prepare PEG-protein grafts, which contained arginylglycylaspartic acid (RGD)-integrin and heparin binding sites with two additional sites for plasmin enzyme induced biodegradation. In another study, RGD cell adhesion peptides were incorporated with polyvinyl alcohol (PVA)-hydrogels [127]. PVA itself does not support cell adhesion. However, it was observed that the PVA-RGD hydrogels induced fibroblast cell adhesion and ECM protein production. Indeed, RGD and various other components, such as peptides derived from laminin Tyr-Ile-Gly-Ser-Arg (YIGSR) and Ile-Lys-Val-Ala-Val (IKVAV) and fibronectin derived peptide Pro-His-Ser-Arg-Asn (PHSRN) have been utilized to control and guide cell behavior in a "dose-dependent" manner by adjusting the ligand density, and therefore signal representation available within the synthetic hydrogel matrix [128,129].

Synthetic co-polymers have also been utilized in a similar way as with the next generation bio-based hydrogels. For example, poly(vinylphosphonic acid-co-acrylicacid) (PVPA-co-AA)-hydrogels were prepared and evaluated as bone-filler materials [130]. PVPA-co-AA binds to divalent calcium ions in bone, and was observed to promote osteoblast adhesion and proliferation. With the synthetic bone-filler hydrogel design, bone mineralization and formation could be induced in bone defects with natural osteoblast function that mimics the bone architecture.

The development of both synthetic and bio-based hydrogels has been increasing in biomedical research and tissue engineering. Currently, both designs have their advantages and limitations. Despite the system design, important factors need to be considered, such as biocompatibility, immunogenicity and biodegradability. For example, biodegradation is an ambiguous property in many hydrogels; i.e. biodegradation processes in the environment do not necessarily occur in the human body. Therefore, the biomaterial needs to be carefully designed based on its potential final use.

4 Biomedical applications of nanofibrillar cellulose

NFC by itself is not digested by human enzymes and does not biodegrade in the human body [131], despite being generally regarded as a biodegradable polymer. However, several studies have been made to evaluate NFC and ANFC cytotoxicity and immunogenicity [132-137]. These studies have shown NFC and ANFC to be well-tolerated biomaterials with no cytotoxic effects, and low potential cytotoxicity even at very high fiber concentrations in NFC. Therefore, these materials can be characterized as biocompatible, with ANFC being slightly safer based on the previous studies. On the other hand, the leaching of non-cellulosic residues arising from the bulk processing and purification methods might lead to immunogenicity of cellulosic materials. It was recently discovered that non-cellulosic residues induced varying polysaccharide-based contaminant levels depending on the fabrication method and source material [138]. This also suggests that the potential leaching of non-cellulosic materials could be easily avoided by utilizing well studied sources and fabrication methods, such as ANFC.

ANFC begins to show gel properties already at a very low 0.09 % (wt/wt) fiber concentration and forms well-structured gels at 0.29 % (wt/wt) concentration [139]. It was also observed, that pH and salt content greatly affected the gel stability. Additionally, high polymer content (3-6.5 %) ANFC hydrogels retain their functionality and ideal gel properties, such as viscoelasticity and shear thinning through rigorous handling methods, such as freeze-drying [140]. The impact of freeze-drying on high polymer content ANFC hydrogel rheology and functionality (e.g. drug release properties) is discussed in more detail in the experimental part of this thesis **(IV)**. NFC and ANFC can be modified chemically relatively easily, which can be used to yield well defined characteristics for intricate applications of a wide variety [141]. It has also been shown that chemical surface modification of NFC did not advance their toxicological profile, which was already observed to be low for cellulose originated nanomaterials [142].

In addition to biocompatibility and outstanding chemical modification capabilities, NFC is relatively inexpensive, has great mechanical properties, and is sustainable and readily available. These properties make cellulose-based biomaterials excellent candidates for biomedical and pharmaceutical applications. For further reference, these applications as discussed are listed on Table 1.

Table 1. *Cell culturing and biomedical applications of plant-derived NFC-based materials.*

Polymer composition	Formulation	Application	Ref.
NFC	Aerogel and film	Wound healing	[143]
NFC-hemicellulose	Composite hydrogel	Wound healing	[144]
NFC*	Surface modified film	Antimicrobial film	[145]
NFC	Cross-linked hydrogel	Antimicrobial hydrogel	[146]
NFC**	Hydrogel bioink	Tailor-made wound dressings	[147]
NFC	NFC wound dressing	Wound healing (clinical study)	[148]
NFC-alginate	Composite hydrogel bioink	Cell-laden ear cartilage scaffold	[149,150]
NFC-carbon nanotube	Conductive hydrogel bioink	Neural tissue engineering	[151]
NFC-polyvinyl acetate	Composite polymer film	Self-softening *in situ* implantation	[152]
Plant cellulose tissue	De-cellularized scaffold	Subcutaneous implantation (*in vivo*-study)	[110]
NFC	Hydrogel	Injectable *in situ* implantation (*in vivo*-study)	[153]
ANFC-chitosan	Hydrogel	Injectable *in situ* implantation (*in vivo*-study)	[154]
NFC	Hydrogel	Injectable hydrogel for localized chemotherapy	[155,156]
NFC-alginate	Composite hydrogel	Suture coating for cell therapy (*ex vivo*-study)	[102]
NFC***	Cross-linked thread	Stem cell delivery (*ex vivo*-study)	[157]
NFC	Hydrogel	3D organoid development	[8,9]
NFC	Hydrogel	3D cell culture scaffold	[121]
NFC	Hydrogel	3D culturing of pluripotent stem cells	[122]
NFC-RS/P	Composite film	Bioadhesive film	[158]
NFC-PEG	Composite hydrogel	Mucoadhesion	[159]
NFC	Aerogel	Gastroretentive drug delivery system	[160]
(A)NFC-polymer†	Composite film	Bioadhesive film	[161]
NFC-chitin	Composite scaffold	Bone tissue engineering	[162]
NFC-gelatin	Composite scaffold	Bone tissue engineering	[163]
NFC-hydroxyapatite	Composite scaffold	Bone tissue engineering	[164]
CNC-GIC††	Composite dental cement	Restorative dentistry	[165]

*Octadecyldimethyl(3-trimethoxysilylpropyl)ammonium chloride modified
**Carboxymethylated and periodate oxidated
***Glutaraldehyde cross-linked
†ANFC- and NFC-Pectin, -mucin and -chitosan composites
††CNC acquired from NFC and reinforced with glass ionomer cement (GIC)

Wound healing

One of the most widely studied biomedical application for NFC (often bacterial cellulose (BC) in this case) is wound healing [166]. Wound healing is a complicated process where the skin repairs itself in various steps [167]. After the skin is damaged, disturbing the blood vessels, platelets attach on the wound site to promote hemostasis. The platelets begin to secrete wound healing and chemotactic factors that, for example, promote fibrin and fibroblast activation and attract macrophages. After hemostasis, inflammation occurs and recruited macrophages remove pathogens, dead and damaged cells and other debris through phagocytosis. New tissue begins to grow through neovascularization, epithelization and granulation tissue formation, where epithelial cells cover the wound and granulation tissue invades the wound space. Finally, during wound maturation, the ECM is reorganized, phenotypic alteration continues until the cells achieve their normal state, and excessive cells responsible for the wound healing processes undergo apoptosis, leaving behind scar tissue that is mostly acellular.

Hydrogel-based wound dressings have several advantages over traditional gauze dressings, such as lower adherence to the wound [168], providing improved wound healing conditions, such as suitable swelling properties and high moisture content [169]. Furthermore, they enhance the removal of damaged and necrotic tissue and debris through adsorption [170], promoting the natural healing processes as described above [171]. BC-based wound healing applications have been extensively investigated and reviewed for commercialized products (XCell, Bioprocess, and Biofill) already in the market [172-175]. However, plant-derived NFC has not received the same level of attention.

In a comprehensive study by Jack et al., plant-derived NFC-based wound dressings were shown to have excellent properties for potential wound healing applications [143]. It was observed that NFC dressings provided an environment with ideal moisture content to promote wound healing. Additionally, it was shown that with different fabrication methods, the porosity and surface roughness could be adjusted. These properties were found to be important in affecting the adsorption and bacterial anti-adhesion capabilities. NFC dressings did not promote bacterial growth nor the secretion of virulence factors. However, hemicellulose reinforced NFC dressings have been shown to promote fibroblast proliferation and viability [144], which could prove beneficial as fibroblasts have a critical role in natural wound healing [176]. Furthermore, NFC matrix supports the fabrication of antimicrobial properties to include an antimicrobial effect [145,146], which could further enhance the wound healing potential of NFC dressings. Indeed, it has been proposed that tailor-made NFC-antibiotic wound dressings could be fabricated with bioprinting [147]. Therefore, as plant-derived NFC shows excellent versatility and have fairly low cost, are non-toxic and biocompatible, they could greatly advance the wound healing research.

In a clinical use, as of writing this thesis, plant-derived NFC has been utilized only once as a wound dressing for the treatment of skin graft donor sites [148]. 9 patients were treated with NFC dressings. 5 patients also received comparative treatment with a commercial product Suprathel® as a reference, which is considered as the current standard treatment by many

clinicians in Finland. NFC was observed to properly attach into the wound bed, and after epithelization, the dressing self-detached without patient discomfort. On one patient the treatment was discontinued due to infection. However, in the wound healing process, the NFC dressings were observed to perform equally with the commercial product Suprathel®. According to their findings, the NFC dressing was biocompatible and epithelization was slightly faster when compared to Suprathel®. However, continuation studies are required to investigate the full potential of plant-derived NFC dressings.

Bioprinting

Recent advances in bioprinting technologies and the use of hydrogels as bioinks has enabled the design and fabrication of finely defined structures [177-179]. As previously mentioned, NFC hydrogel bioinks have been utilized in mimicking corneal ECM and in the fabrication of tailor-made wound dressings [114,147]. Another excellent example of a carefully designed use of 3D printing is the anatomically correct human ear cartilage tissue structure with NFC-based bioink [149]. MRI and CT acquisitions were used as the blueprints for cartilage fabrication. Human nasoseptal chondrocytes were used to evaluate cell bioprinting and bioink *in vitro* biocompatibility. The bioink preparation and bioprinting processes initially lowered cell viability. However, after 7 days of cell culture in the constructs, the viability increased; therefore, the bioprinted constructs themselves did not significantly affect cell viability. These systems could be utilized in tissue repair where the construct can be fabricated to resemble the original tissue before the tissue defect, such as the human ear. Later it was investigated that the same constructs support human primary nasal chondrocyte redifferentation, preservation of their phenotype and induced the secretion of cartilage specific ECM components [150]. Therefore, resembling the biological formation and growth of natural human cartilage. As the cells are harvested from the patient him-/herself, the immunogenicity of the graft could potentially be reduced while maintaining the cartilage function and structure to repair the tissue defect. Another example of NFC-based bioprinting is the fabrication of a conductive neural tissue scaffold [151]. NFC and carbon nanotube composite bioinks were prepared and 3D printed as a scaffold to guide neural cell behavior. It was observed that human-derived neuroblastoma cell attachment, growth, proliferation and differentiation was affected by the conductive NFC-based scaffolds, while the constructs had minimal effect on cell viability. Such systems provide a good basis for potential improvements in neural tissue engineering, which could further the treatment of currently incurable neuronal diseases, such as Parkinson's or Alzheimer's disease.

In situ implantation

Another example of a cellulose-based material as a biomedical device in neural tissue engineering is cellulose nanocrystal and polyvinyl acetate (CNC-PVAc)-composite, which functions as an external stimuli-responsive implantable biomaterial [180]. CNC-PVAc changes its mechanical properties *in situ* when exposed to artificial cerebrospinal fluid. The matrix in

its higher tensile strength state (i.e. dry) enables the implantation procedure and then softens when in contact with a fluid (i.e. water) to mimic the mechanical properties of the surrounding tissue, such as the brain [181]. The design aspect for such reversible stimuli-responsive biomaterials originated from sea cucumbers, which are able to change their dermal stiffness "on command" [182]. The mechanism was later found and is suspected to be mediated by a protein appropriately named as "softenin" [183]. Due to these properties, CNC-PVAc devices were implanted into the cerebral cortex of 8 Sprague-Dawley rats [152]. It was shown that the devices transformed to mimic the mechanical surroundings of the cortical tissue. The advantages of *in situ* softening CNC-PVAc implants are reduced inflammatory responses and glial scarring when compared to conventional stiff implants [184,185]. *In situ* implantation can also be achieved with NFC hydrogels through injections. While external environmental triggers can induce hydrogel gelation, as explained earlier, the response process is often too slow [186], which is impractical for clinical use. The shear-thinning properties, however, enable the hydrogel to regain their strong viscoelastic gel structure immediately after the high shear forces subside from the injection process. In the experimental part of this thesis, the *in situ* injectability aspect of NFC hydrogel [153] is discussed in more detail and studied *in vivo* **(II)**. Overall, only a few studies have investigated the potential use of plant-derived NFC in implantation as BC is generally considered superior over NFC [187]. However, while plant-derived cellulose materials are underrepresented, the versatility and much better availability over BC has increased its interest in the biomedical field [188].

Bioadhesion

Bioadhesion is another recently emerged topic for NFC-based hydrogels. Bioadhesion is acquired through a contact between a hydrated biological layer and the application surface. Such applications can be used for controlled drug delivery or local/topical drug administration. In a recent study, colon specific drug delivery was achieved with the use of NFC reinforced resistant starch/pectin (RS/P) films [158]. BC and NFC were compared, and it was observed that NFC provided far better properties as a reinforcing material, such as stronger bioadhesion, improved mechanical properties and interaction with the RS/P matrix. Strong bioadhesion was acquired, and it was shown that NFC had a better control over the release rate of methotrexate than BC. Overall, NFC was shown to be very effective as a reinforcing material to improve the functionality and properties of RS/P films. In another study, different nanofibrous blends with various polymers were investigated in terms of bioadhesion [159]. It was observed that nanofibrillated blends acquired from carboxymethylcellulose (CMC) and PEG had the highest potential for a bioadhesive application. Other investigated blends were PEG-alginate, PEG-polyacrylic acid and PEG by itself. Also, in a recent study light-weight NFC-based aerogels were prepared for a bioadhesive drug release application [160]. The aerogels were shown to have great mechanical properties and enhanced *in vivo* bioavailability of bendamustine hydrochloride in Wistar rats. Bioavailability was enhanced by a factor of 3.25 and 5.66 when compared with oral drug solution and intravenous injection respectively, with a T_{max} of 4 h for the aerogel formulation vs. the 1 h of oral drug solution. Additionally, C_{max} values were slightly

lower for the aerogel formulation than oral drug solution. Therefore, with bioadhesive formulations, these applications are able to increase the bioavailability of drug compounds (especially poorly soluble ones) by increasing the retention time, and additionally avoiding adverse effects. NFC and ANFC were also investigated as a local drug releasing bioadhesive fomulations **(V)** in this thesis. This study is discussed in more detail in the experimental part.

Short summary

Plant-derived NFC has great potential in the biomedical field as a versatile biomaterial. The versatility of NFC enables the design of various biomedical applications, which could improve current treatment methods and provide novel alternative solutions to several challenges. Currently, NFC is underrepresented in clinical and *in vivo* animal studies when compared to BC. However, the supporting information built upon the NFC research as a biomaterial, warrants more investigations into the use of NFC in practical biomedical applications.

5 Aims of the study

The objective of this thesis was to investigate different aspects of NFC-based hydrogels as functional biomaterials for pharmaceutical and biomedical applications, such as 3D cell culture, *in situ* implantation, suture coating material for cell delivery, controlled drug release and bioadhesive films. The characteristics and performance of these applications were evaluated *in vivo* and *in vitro*. Specific aims were as follows:

1) Fabrication of functional hydrogel biomaterials. **(I-V)**

2) Characterization of nanofibrillar cellulose as a cell culture biomaterial and delivery device for therapeutic use. **(I-V)**

3) Modification and optimization of hydrogel biomaterials. **(II-V)**

4) Functionality of fabricated biomaterials as active materials and potential applications. **(I-V)**

6 Experimental

6.1 Materials (I-V)

Nanofibrillar cellulose (NFC) and anionic carboxylated NFC (ANFC) stock hydrogels: 1.7 % NFC **(I)**, 1.6 % NFC (GrowDex®) **(II)**, 1.47 % NFC (GrowDex®) **(III)**, 3.2 % and 6.8 % ANFC (FibDex™) **(IV)** in addition to 1.5 % NFC (GrowDex®) and 2.7 % ANFC **(V)** were provided by UPM, Finland. MaxGel™ (MG), ExtraCel™ (EC), HydroMatrix™ (HM) and PuraMatrix™ (PM) were purchased from Sigma-Aldrich, Glycosan biosystems, Sigma-Aldrich and BD Biosciences, respectively. Stannous chloride dehydrate solution (Angiocis®) was obtained from IBA Molecular, Belgium. The cocaine analogue ^{123}I- β-CIT was purchased from MAP Medical Technologies Oy, Finland. Human serum albumin (HSA) and bovine serum albumin (BSA) were purchased from Sigma-Aldrich, Finland. ^{99m}Tc-pertechnetate was used to label NFC (^{99m}Tc-NFC) and HSA (^{99m}Tc-HSA). Polyethylene glycol 6000 was purchased from Fluka, Switzerland. Sodium alginate was obtained from Sigma-Aldrich (W201502, Finland). Surgical sutures (Velosorb™ Fast 3-0) were purchased from Covidien, USA. Metronidazole (MZ), Nadolol (NAD), FITC-dextrans (tested molecular weights) and trehalose were obtained from Sigma-Aldrich. Ketoprofen (KETO) was purchased from Orion Pharma and Lysozyme (LZ) from Roche, Germany. Chitosan oligosaccharide lactate (Sigma-Aldrich, USA), mucin from bovine submaxillary gland (EMD Millipore, USA) and pectin from apple (Sigma-Aldrich, China) were used as mucoadhesive polymers in bioadhesive films. Microcrystalline cellulose (MCC Avicel PH200) was purchased from FMC BioPolymer, Ireland. Porcine stomach (Type-II) and bovine submaxillary gland (Type-I) mucins were acquired from Sigma-Adrich, USA.

Cell lines

Human liver progenitor cells, HepaRG, were cultured in William's E medium supplemented with 10% FCS, 1 % penicillin/streptomycin, 5 µg/ml insulin, and 5 x 10^{-5} M hydrocortisone hemisuccinate. Human ARPE-19 cells (ATCC® CRL-2302™, USA), were cultured in DMEM-F12 supplemented with with 10 % fetal bovine serum (FBS), 2 mM L-glutamine, 1 % penicillin/streptomycin **(I)**.

Human hepatocellular carcinoma cells, HepG2 (ATCC® HB-8065™, USA) and human adenocarcinoma-derived endothelial cells, SK-HEP-1 (ATCC® HTB-52™, USA) were cultured in high glucose DMEM supplemented with 10 % FBS, 2mM L-glutamine, 1 % penicillin/streptomycin and 100 mM sodium pyruvate **(I, III)**.

Human squamous cell carcinoma cells, TR146 (Sigma-Aldich, 10032305, ECACC, UK), were cultured in DMEM-F12 supplemented with 10 % FBS and 2 mM GlutaMAX™ (Thermo Fisher, USA) **(V)**.

All cells were cultured at 37°C and 5 % CO_2. After reaching 70-80 % confluency, the cells were passaged **(I, III, V)**.

6.2 Nanofibrillar cellulose biomaterial fabrication (I-V)

All NFC and ANFC hydrogels were purified and homogenized from aseptically collected wood pulp with sterile machinery by UPM. Pulp material was diluted in sterile ultra-high quality water prior to fibrillation. Stock hydrogels obtained from fibrillation were autoclaved at 121 °C for 20 minutes before cell culturing **(I)**. Afterwards, sterile stock hydrogels were obtained directly from UPM **(II-V)**.

1 % ^{99m}Tc-NFC hydrogels were prepared with a stannous chloride reduction method for *in vivo* implantation. Radiolabeling efficiency and stability were evaluated with ITLC-SG chromatography plates (Agilent Technologies, Santa Clara, CA, USA) in methylethylketone (MEK) solvent system. Samples were collected in standard RIA tubes and measured with RiaCalc. WIZ, (Wallac 1480 WIZARD® 3" gamma counter, Finland). 5:1 ratio of 1 % NFC and saline solutions of ^{99m}Tc-HSA, ^{123}I-NaI and ^{123}I-β-CIT were prepared separately for *in vivo* SPECT/CT imaging **(II)**.

NFC-alginate (NFCA) hydrogels were prepared for cell culture and surgical suture coatings. NFCA hydrogels, composing of 8 % (wt/vol) sodium alginate and 1.35 % (wt/vol) NFC were cross-linked with 20 mM barium chloride (Sigma-Aldrich, Finland) and 68 mM calcium chloride (Riedel-de-Haen, Germany) solutions and left to settle for 24 h. For cell co-culturing, 1 mg/ml type-I collagen (Cultrex®, Trevigen USA) was used to enhance cell adhesion on NFCA surface **(III)**.

3 % and 6.5 % freeze-dried ANFC aerogels were prepared by snap freezing in liquid nitrogen and transferred to FreeZone 2.5 (LabConco, USA) and freeze-dried in 70 mTorr at -52 °C for 29 h **(IV)**. Prior to diffusion studies and rheological characterization, the freeze-dried samples were rehydrated and redispersed with ultra-pure water 1.1 % ANFC cross-linking was performed with cationic aluminum sulfate hydrate (Sigma-Aldrich, USA), calcium chloride (Sigma-Aldrich, Japan) and iron(III) nitrate nonahydrate (Sigma-Aldrich, Germany). Solid powders were dissolved in ANFC and left to settle for 48 h. Concentrations were 2.5 mmol/kg for Al^{3+}, 4.4 mmol/kg for Ca^{2+} and 2.2 mmol/kg for Fe^{3+} **(IV)**.

Bioadhesive films were prepared by mixing 1 % NFC and ANFC hydrogels with mucoadhesive polymers mucin, pectin or chitosan with 2:1 cellulose:mucoadhesive polymer fiber content ratio (wt/wt). 10:1 and 1:1 ratios were also used in the bioadhesion measurements. Hydrogel mixtures were dried in plastic petri dishes and polylactic acid/teflon molds with a diameter of 4 cm in both systems at 45 °C for 18 h. Films were prepared with and without MZ. MZ content was set to 10 % of dry polymer mass **(V)**.

6.3 Characterization of NFC hydrogel-based biomaterials (I-V)

Morphology

Scanning electron microscopy (SEM) for NFC hydrogels were performed with JEOL JSM-7500FA field-emission SEM. Fibril diameters were measured from 300 single fibrils from SEM images with the built-in image analysis software. Cryo-transmission electron microscopy was performed with field emission cryo-electron microscope (JEOL JEM-3200FSC) operated at 300 kV. Micrographs were recorded with Gatan Ultrascan 4000 CCD camera **(I)**.

Freeze-dried ANFC aerogel morphology was investigated with SEM (Quanta FEG250, FEI Company, USA). Surface and cross-sectional structures were imaged from fixed samples sputtered with platinum (Agar Scientific Ltd., UK) **(IV)**.

NFC and ANFC film morphology with and without mucoadhesive polymers was imaged with SEM (Quanta FEG250, FEI Company, USA). Films were fractured and cross-sectional micrographs were taken with surface images. Film pieces were fixed and sputtered with platinum (Agar Scientific Ltd., UK) **(V)**.

Optical properties

Absorbance of 0.5 % NFC (wt/wt) was measured with UV spectrometer (QuantaMaster™, Photon Technology International) at 300-550 nm and fluorescence spectra acquired at excitation wavelenghts 405, 488 and 560 nm, using Hoechst 33258, FITC-dextran 70 kDa and rhodamine 123 as positive controls and purified water as a negative control **(I)**.

Thermogravimetry

Freeze-dried ANFC aerogel residual water content was measured with TGA 850 (Mettler-Toledo, Switzerland). Heating rate was set to 10 °C/min (25-240 °C) in nitrogen atmosphere. Mass loss (%) parameter was used to evaluate evaporated moisture **(IV)**.

Differential scanning calorimetry

Thermal analysis of ANFC aerogels were performed with DSC 823e (Mettler-Toledo, Giessen, Germany). Sealed aluminum pans with closed lids were used and heated at a rate of 10 °C/min (25-200 °C) in nitrogen atmosphere. Analysis was performed with STARe software (Mettler-Toledo, Giessen, Germany). Measurements were performed after one month of storage in silica at 20 °C **(IV)**.

Thermal analysis of NFC and ANFC films were performed with DSC 823e (Mettler-Toledo, Giessen, Germany). Aluminum pans with perforated lids were used and heated at a rate of 10 °C/min (25-220 °C) in nitrogen atmosphere. Analysis was performed with STARe software (Mettler-Toledo, Giessen, Germany) **(V)**.

Fourier-transform infrared spectroscopy

FTIR spectroscopy was performed using a Vertex 70 FTIR spectrometer (Bruker Optik GmbH, Germany) with a MIRacle^{TM} single reflection ATR crystal (Pike Technologies, Inc., USA). The analytical range of measurements was 650-4000 cm^{-1} with a spectral resolution of 4 cm^{-1}. Each spectrum was collated as an average of 64 scans with three spectra recorded for each sample **(V)**.

Raman spectroscopy

Raman spectroscopy performed for NFC and ANFC films with a Raman RXN1 spectrometer (Kaiser Optical systems, Inc., USA), equipped with a PhAt probe and a 20-mW laser source operating at 785 nm. Each spectrum was recorded as an average of three scans with an integration time of 1 s. For NFC and ANFC control films the parameters were ten scans with 3 s integration times. Elevated baselines of the spectra were removed and analyzed with Opus software (Bruker Optik GmbH, Germany). Before analysis the spectra were normalized by SNV transformation and mean centering by PCA (SIMCA software, Sartorius Stedim Biotech, Sweden) **(V)**.

Rheology

Rheological properties of NFC were investigated at room temperature using a rotational rheometer (AR-G2, TA instruments, UK) with 28 mm vane blade geometry and 30 mm cylindrical cup. Frequency sweeps were performed with dynamic oscillatory mode at a strain of 0.1 %. Viscosity measurements were performed at a shear stress range of 0.01-100 Pa **(I)**.

Rheological properties of NFCA composite hydrogels were measured with Haake™ Viscotester™ iQ Rheometer (ThermoFischer, Germany) with 2° cone-plate geometry (35 mm in diameter with 0.1 mm gap). Frequency sweeps were performed with a constant amplitude on a range of 0.1-20 Hz. Viscosity was measured with controlled rate mode ranging 0.1-1000 1/s **(III)**.

1.1 %, ANFC hydrogels (cross-linked and non-cross-linked), 3 % and 6.5 % (hydrogels and re-hydrated hydrogels) were measured with Viscotester™ iQ Rheometer. Plate-plate geometry (35 mm in diameter with 1 mm gap) was used. Frequency sweeps were made with a constant amplitude on a range of 0.1-20 Hz. Viscosity was measured with controlled rate mode ranging 0.1-1000 1/s **(IV)**.

All rheological measurements were made in triplicate with a Peltier temperature control system set to 25°C **(I, III)** or to 37 °C **(IV)**.

Tensile strength of films

20 x 2 mm films were fixed on a measuring table in a humidity controlled tensile tester (Kammrath & Weiss GmbH, Germany) with a tensile/compression module of 5 kN and a 100 N load cell. Young's modulus (YM), tensile strength, elongation and toughness values were measured and calculated form the average of the strain-stress curves. YM was obtained from the slope of the elastic region and tensile strength and elongation obtained from the point of fracture. Toughness was estimated as the work-of-fracture, by integrating the area under the stress curve **(V)**.

Swelling of films

1 cm^2 pieces of cellulose:mucoadhesive polymer films were submerged in phosphate buffer (6.8 pH) for 15 s and 5 h at room temperature. The weight of the film pieces was measured prior and after hydration. Afterwards the films were dried at 45 °C for 20 h and measured again. Hydration % and mass loss % were calculated. Additionally, pieces containing MZ were immersed for 30 min and film dimensions (thickness and area) were measured prior and after hydration with Image J software (National Institutes of Health, Bethesda, USA) **(V)**.

Toxicity

Toxicity study was performed to evaluate NFC/ANFC-mucoadhesive polymer containing film cytotoxicity. TR146 cells were incubated with 0.5 cm^2 film pieces for 24 h. Film pieces were removed and cell proliferation assay (alamarBlue®, Thermo Fisher, USA) was performed with Varioskan LUX microplate reader (Thermo Fisher, USA) at 565 nm excitation and 585 nm emission wavelengths **(V)**.

6.4 Functionality of hydrogel biomaterials (I-V)

Implantation

NFC hydrogel injectability was evaluated with ARPE-19 cell seeding efficiency test. $2.5*10^4$ cells were seeded in 200 µl of 1.7 % NFC (wt/wt) and cultured for 48 h on a standard 96-well cell culture plate. After incubation, the hydrogel samples with the cells were gathered with 1 ml syringes and transferred into empty wells on a new 96-well plate and cultured for 24 h. Various needle sizes (20-27G) were tested when transferring the samples. Cell proliferation assay (alamarBlue®, Thermo Fisher, USA) was performed with Varioskan Flash microplate reader (Thermo Fisher, USA), excitation and emission wavelengths were 565 nm and 585 nm respectively **(I)**.

NFC implants (200 µl) were injected in the pelvic region of 20 female BALB/c mice subcutaneous tissue with ^{123}I-NaI, ^{123}I- β-CIT or ^{99m}Tc-HSA mixed with the NFC. All mice received a dose of 50-60 MBq per implant. SPECT/CT images were acquired with a small animal scanner (NanoSPECT/CT®, Bioscan, USA) equipped with multipihole apertures.

Image reconstruction was performed with HiSPECT NG software (Scivis GmbH, Germany) and fused with the CT acquisitions (InVivoScope™ software, Bioscan Inc., USA). Volumes of interests were drawn over thyroid glands, stomach, left kidney, heart, striatum and the site of injection. Radioactive decay was corrected and normalized to the time of injection. Finally, ^{99m}Tc-HSA clearance models were made with Phoenix® WinNonlin® (Pharsight, Mountain View, USA) **(II)**.

Diffusion and drug release studies

20, 70 and 250 kDa FITC-dextrans were used to model nutrient and macromolecule transportation in NFC. 300 µl of NFC hydrogel was pipetted as an even layer (3 mm thick) on the apical compartment of 12-well transwell plate and 200 µl of 125 µg/ml FITC-dextrans with different molecular weights in PBS were added on top of the hydrogel layer. Samples were collected from the basolateral compartment every 15 min for 2 h and afterwards every 30 min for 4 h, and replaced with PBS. Samples were measured with Varioskan Flash microplate reader (Thermo Fisher, USA), with excitation wavelength of 490 nm and emission wavelength of 530 nm **(I)**.

Test compounds KETO, MZ, NAD, LZ, 10 kDa FITC-dextran and BSA were used to model drug diffusion and release in ANFC hydrogels. 1.1 %, 3 % and 6.5 % (wt/wt) hydrogels were thoroughly mixed with test compounds as monolithic solutions. Freeze-dried ANFC hydrogels with test compounds KETO, MZ, NAD and BSA contained cryprotectans PEG6000 and trehalose. 1.07 g of sample (hydrogel or re-hydrated hydrogel) was placed in a mold with 1.33 cm^2 flat surface area submerged in test buffer (pH 7.4). Hydrogels were kept at constant magnetic stirring and samples collected for up to 144 h. Quantification for KETO and NAD were measured with ultra-performance liquid chromatography (Acquity UPLC, Waters, USA) at the 255 nm and 215 nm wavelengths respectively. FITC-dextran was measured with Varioskan Flash at 490 nm excitation and 520 nm emission wavelengths. The absorbance of MZ and LZ were analyzed with Cary 100 UV-vis spectrophotometer (Varian Inc., USA) with the wavelengths of 320 nm and 280 nm respectively. BSA was quantified with Bio-Rad protein assay (Bio-Rad, USA) based on Bradford dye-binding method **(IV)**.

The release of MZ was measured from 1 cm^2 pieces cut from NFC/ANFC-mucoadhesive polymer containing films prepared in polylactic acid/Teflon molds. Pieces were submerged in release buffer (pH 6.8) and kept under constant magnetic stirring. Samples were collected for up to 30 min, filtered and diluted prior to UPLC analysis. Analysis was performed with ultra-performance liquid chromatography (Acquity UPLC, Waters, USA) at the 317 nm wavelength. **(V)**.

Cell culture

HepG2 and HepaRG 3D cell culturing in commercial biomaterials MG, EC, HM and PM was performed according to the manufacturer's instructions, and compared to 3D NFC hydrogel

cell cultures in 0.1-1.2 % NFC (wt/wt). Hepatic spheroid formation, morphology, vialibity and functionality were evaluated. Cell viability (alamarBlue®), total protein analysis (BCA Protein Assay Kit, Pierce Biotechnology, USA) and albumin secretion (Human Albumin ELISA kit, Bethyl Laboratories, USA) were all performed according to the manufacturers instruction. Confocal microscopy was used to determine hepatic spheroid formation and morphology with Alexa Fluor 594 phalloidin (Invitrogen A12381), Hoechst 33258 and fluorescein diacetate (Molecular Probes®, USA), using Leica TCS SP5 II HCS A (Leica Microsystems, Germany) **(I)**.

HepG2 cells were encapsulated within the NFCA matrix (1043 cells/µl) as a model for 3D culture and SK-HEP-1 cells were cultured on the NFCA surface to model 2D monolayer culture. Live/Dead confocal imaging was performed with Leica TCS SP5 II HCS A, using fluorescein diacetate and propidium iodide (Molecular Probes®, USA) for live and dead cells respectively. Cell co-culture dual staining and confocal imaging was performed with CellTracker™ Green CMFDA and CellTracker™ Red CMTPX (Molecular Probes®, USA) for SK-HEP-1 and HepG2 cells respectively **(III)**.

Surgical suture coatings

NFCA hydrogels with HepG2 cells were prepared in a syringe. A commercial biodegradable suture (Velosorb™ Fast) was fed through the needle orifice and NFCA-HepG2 mixture extruded on top of the suture as a thin layer ($1.5*10^4$ cells/cm/suture). Sutures were cross-linked and sewn through pig liver segments and analyzed with confocal microscopy (Leica TCS SP5 II HCS A). Suturing performance of NFCA coated sutures was investigated in an *ex vivo* study with BALB/c mice and a Wistar rat. Several types of soft tissue (intestine, skin, liver, spleen, muscle and testis) were sutured and photographed for evaluation **(III)**.

Bioadhesion

600 g direct compression Type-II mucin/MCC discs with a 4:1 ratio (wt/wt) and 1 % type-I mucin solutions were prepared for texture analysis (TA.XT plus with a 5 kg load cell, Stable Microsystems Ltd.,UK). The disc was attached onto a 10 mm diameter TA.XT plus probe and wetted with type-I mucin solution to simulate the mucosa. Films prepared in Petri dishes were fixed under the probe, which was lowered at a rate of 0.30 mm/s and a force of 100 g was applied on the film surface for 15 s. Peak adhesion force (N) was measured when the probe was detached from the film with a retraction speed of 0.30 mm/s. The force required to detach the probe was recorded and analyzed with Exponent software (Stable Microsystems Ltd., UK) **(V)**.

7 Results & discussion

NFC-based biomaterial characteristics and applications

In the following experimental part, NFC-based hydrogels were prepared and utilized in various pharmaceutical and biomedical applications. The main focus was on the applications and their functionality. However, some discussion is related to the biomaterial characteristics, when appropriate. More detailed information about the hydrogel properties can be found in the publications.

Native NFC rheology and 3D cell culture (I)

The entangled cellulose fibrils formed stable viscoelastic networks typically characterized by a high storage modulus (G') with a lower loss modulus (G''), while being relatively independent of angular frequency [189], as shown in Figure 4A. It was noticed that the G' values in relation to material stiffness were comparable to a relatively low (1-2 mg/ml) collagen matrix, which has been reported to support cell motility [190]. As a pseudoplastic material, NFC was observed to follow shear-thinning behavior with increasing shear stress (Figure 4B).

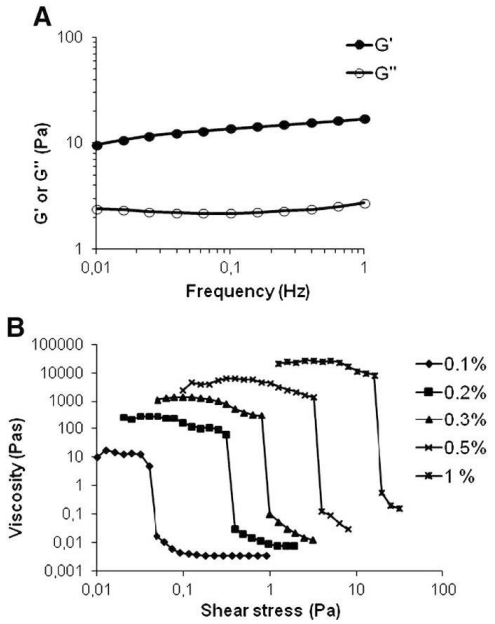

Figure 4. *The viscoelastic behavior and shear-thinning of NFC. A) The high ratio of G' and G'' is relatively independent of angular frequency. B) The shear-thinning effect of different concentrations of NFC.*

Shear-thinning is an important property of NFC, as it enables hydrogel injectability. The viability of ARPE-19 cells was examined after the transfer of cell culture through a syringe with various needle sizes up to 27G (inner Ø: 210 µm). The cells remained viable after syringe transfer, independent of the inner needle diameter (**I**, Figure 4). Indicating that NFC hydrogel protects and is able to encapsulate the cells safely against the high shear forces of the transfer. The shear-thinning effect in the syringe can be described by applying pressure to the hydrogel (plunger), which forces the material through the small needle, exposing it to high shear stress. NFC transitions into a fluid-like state, which prevents the needle from clogging, therefore enabling injectability. Additionally, after the applied force has been lifted, NFC recovers to its original viscoelastic state, due to its thixotropic properties. Therefore, the shear-thinning effect is reversible, which can be exploited in applications requiring injections and fast gelation responses.

NFC was observed to have high viscosity even at below 1% fiber content (Figure 4B). The aqueous media bound within the network is therefore very high, which enables the transportation of nutrients, waste materials, metabolites and other factors secreted by the cells. Therefore, the permeation of various large molecules (20-70 kDa dextrans) was investigated through a layer of NFC hydrogel (**I**, Figure 6A). It was observed that the calculated diffusion coefficient values of dextrans (**I**, Figure 6B), followed similar diffusion behavior as natural proteins in ECM [191]. This further suggests that the NFC microenvironment resembles the ECM of natural soft tissue in terms of viscoelasticity and molecular diffusion. Additionally, the intrinsic high viscosity of NFC polymer fiber network was able to retain the cells as a suspension within the matrix (Figure 5A). To evaluate the 3D cell culturing capabilities of NFC, hepatic cell morphology and functionality was investigated and compared with various commercially available cell culturing hydrogels. It was observed that HepG2 and HepaRG cells were able to form multicellular spheroids, which is typical for hepatic morphology (Figure 5B). The hepatic 3D structure of the spheroids was shown to express cell polarity, often associated with bile canaliculi formation [192]. Hepatic differentiation of hepatic progenitor cells HepaRG and functionality of HepG2 were examined by measuring cell albumin secretion. Albumin secretion increased in HepaRG-NFC cultures during the study period (Figure 5C), which indicates hepatic cell differentiation [193]. In HepG2-NFC cultures, the albumin secretion remained relatively steady (Figure 5D), which is a common biomarker for HepG2 hepatic functionality. Furthermore, it was observed that NFC did not induce cytotoxicity in HepG2 and HepaRG cultures (**I**, Figure 6E-H).

According to these findings, NFC is an excellent platform for a functional 3D cell culture with equal or enhanced performance when compared to commercial hydrogels MaxGel™, ExtraCel™, HydroMatrix™ and PuraMatrix™. NFC hydrogels were not cytotoxic and are free of any animal-based materials. NFC possesses inherent similarities with natural soft tissue and forms gels spontaneously without the need for chemical or any other external stimuli, such as heat. NFC shear-thinning enables hydrogel injectability, which suggests a potential for a wide variety of implantation and cell delivery applications.

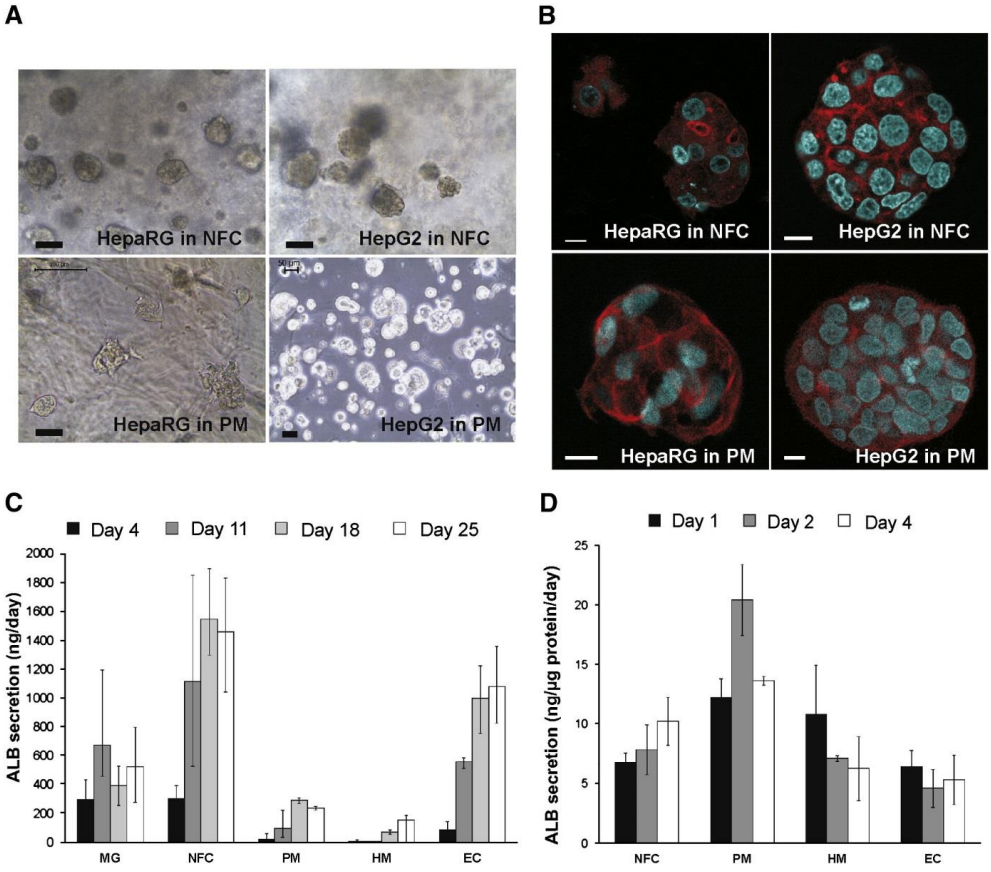

Figure 5. *Morphology and functionality of the hepatic cell cultures of HepaRG and HepG2. A-B) Hepatic cell cultures, filamentous actin (red) and nuclei (blue), in NFC and PuraMatrix™ indicate typical spheroid formation with cell polarization. C) HepaRG differentiation as shown by increase of albumin secretion during the study. Cultures in NFC and ExtraCel™ were observed to express enhanced albumin secretion when compared to other hydrogels. D) HepG2 albumin secretion in NFC cultures were equal with HydroMatrix™ and ExtraCel™, while PuraMatrix™ showed enhanced albumin levels.*

Native NFC as an injectable implant (II)

The injectability of plant-derived NFC was investigated and the results indicate that it could be used in implantation applications **(I)**. NFC does not require any external sources to invoke a gelation response, as it occurs spontaneously. Indeed, many hydrogels require a chemical agent, or external stimuli from the environment to achieve gelation. However, these processes are often very slow [186], or require toxic cross-linkers [194] and as such are not practical as an actual biomedical application. Therefore, NFC hydrogels were used *in vivo* to study its functionality as an *in situ* drug releasing implant with the use of a small animal dual imaging modality single-photon computed tomography/X-ray computed tomography device (SPECT/CT) (Figure 6). The aim was to investigate the drug release properties of NFC on small (^{123}I-NaI and ^{123}I- β-CIT) and large (^{99m}Tc-HSA) molecules *in vivo*. Specifically, the controlled release properties of the large compound ^{99m}Tc-HSA. Additionally, it was studied if the injectable implant remains intact after administration, as the mice were allowed to move freely between the image acquisitions.

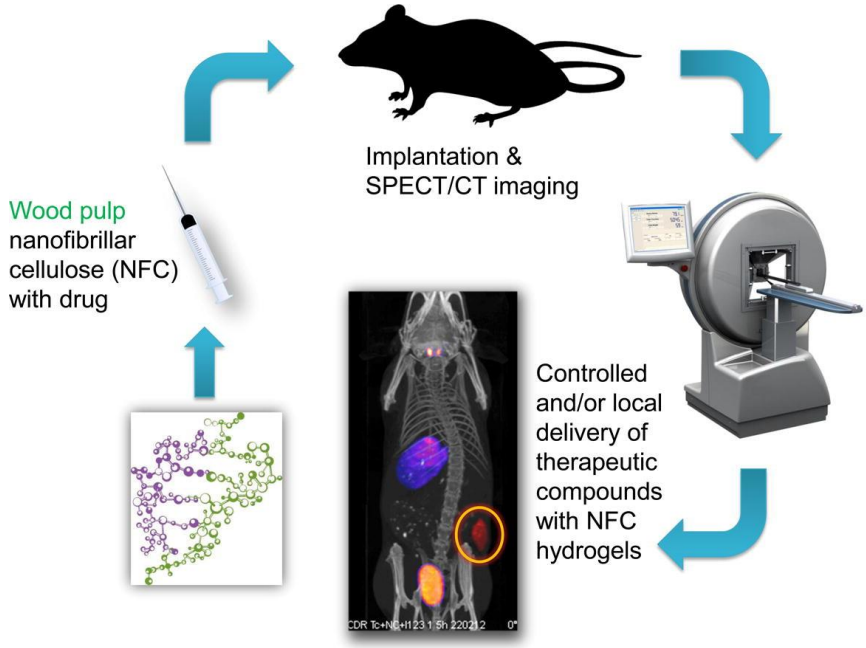

Figure 6. *NFC hydrogels as in situ drug releasing injectable implants. NFC is encapsulated with a drug compound, which is then transferred into a syringe. The injection is given to the study animals and imaged with SPECT/CT. Both the drug compound and NFC can be traced simultaneously with a radiolabel, to investigate the functionality of the implant and evaluate its drug release properties.*

The NFC hydrogels were mixed with either ^{123}I-NaI, ^{123}I- β-CIT or ^{99m}Tc-HSA and given as a subcutaneous injection to 20 BALB/C mice. Additionally, NFC was radiolabeled and evaluated in dual tracing images with the drug compound (Figure 7). It was observed that the small molecular drug compounds ^{123}I-NaI and ^{123}I- β-CIT were released rapidly as was noticed from the expanse of activity around the implant 15 min post administration. Additionally, the site of injection did not show any activity 5 h post administration. It was observed that ^{123}I- β-CIT accumulated faster in its target organ (striatum) when given with saline control injections, indicating a slightly slower release from the NFC hydrogel. However, the difference in accumulated dose was almost negligible. With the larger compound ^{99m}Tc-HSA, 41 % of the injected dose had been released from the NFC hydrogel 5 h post administration. A 2-fold difference was observed in the clearance rate of ^{99m}Tc-HSA between NFC and saline sites of administration, with the site of NFC being the slower one. This agrees with the finding that the NFC matrix hinders the diffusion of large compounds **(I)**.

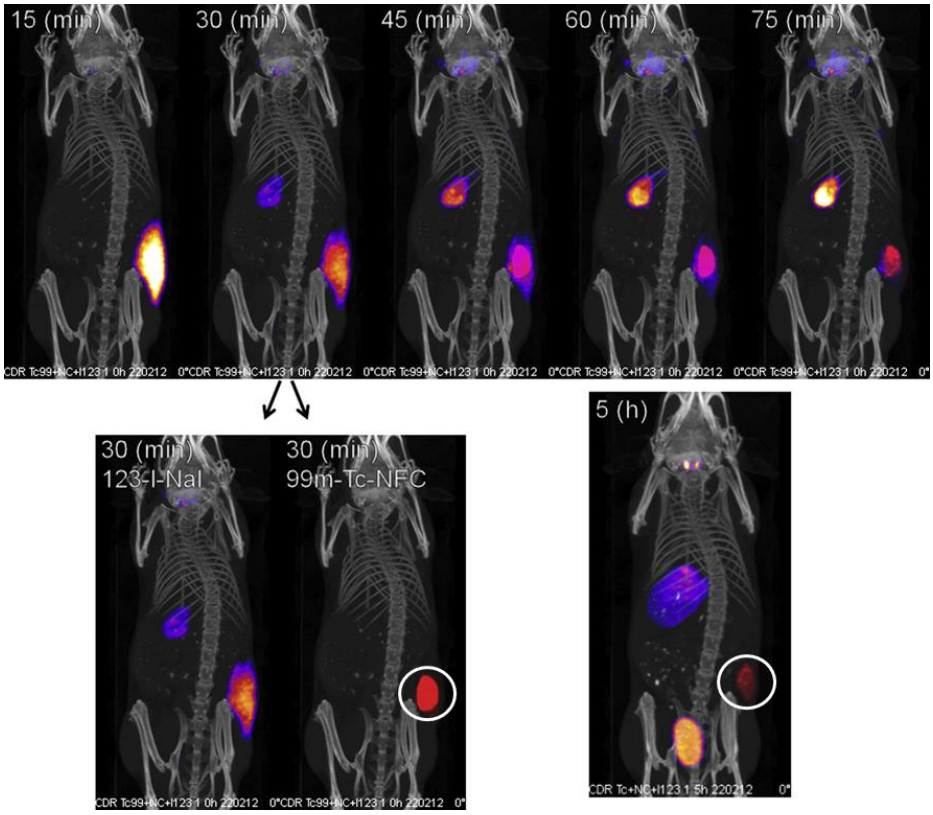

Figure 7. *^{123}I-NaI and ^{99m}Tc-NFC dual trace imaging. The release of ^{123}I-NaI from ^{99m}Tc-NFC was rapid, as most of the dose has been absorbed from the site of injection at 75 min. White circles indicate the NFC implant, which has remained intact during the study period.*

During the study period, the mice could move freely in their habitats in between the first set, 5 h and 24 h acquisitions. It was observed that the NFC implants remained intact without any signs of deformation or disintegration after the injection. This indicates that the stress caused by normal movement is not enough for NFC to transition into the fluid-like state, as was described with high shear forces **(I)**.

NFC did not inhibit the release of small molecules when compared with saline injections, therefore it can be concluded that no apparent interactions were between the small molecules and NFC. The large molecule ^{99m}Tc-HSA showed similar inhibition in diffusion as was observed earlier **(I)**. Therefore, large molecule release rate can be controlled with adjusting the NFC fiber content **(I, IV)**. Local or rapid drug release can be achieved with small molecules. For example, NFC hydrogels could be spread over a wound with a fast-acting drug molecule to achieve immediate treatment, especially if the hydrogel is enhanced with a bioadhesive element **(V)**.

Overall, the advantages of NFC implants are injectability and adjustability in terms of fiber content. NFC self-gelation occurs immediately after the high shear forces have been lifted, therefore no external factors are required to achieve the gel-transition functionality. Potential toxicity from cross-linking residues can be avoided completely and the slow gelation response is not an issue. Therefore, the system is very simple and easy to use, which makes it readily available for any number of *in situ* implantation applications. However, some challenges remain regarding the application. For example, NFC is not biodegradable in the human body and despite the chemical modification capabilities, NFC self-degradation *in vivo* has not been extensively studied or explicitly shown. Fortunately, these challenges can be influenced to some effect. It was shown that cell cultures could be transferred with a syringe and a needle **(I)**. Therefore, in the removal of subcutaneous implants, it could be possible to utilize a similar technique, i.e. the implant could be removed as it was administered, by injection. Alternatively, an additional injection could be administered containing enzymes responsible for cellulose degradation. The metabolic products of NFC (mostly glucose) and the enzymes themselves have been shown to not induce cytotoxicity [122]. Despite these possibilities, NFC requires additional studies as an injectable implant to fully utilize its potential. Therefore, it is suggested that currently NFC-based materials are utilized in easily accessible locations, such as under the skin **(II)**, as skin patches in e.g. wound healing, or outside of the body, such as the gastro-intestinal (GI)-tract **(III, V)**.

NFC-alginate (NFCA) polymer composites in cell delivery (III)

NFCA composite polymers were prepared to investigate whether it would be a potential system for cellular delivery. Alginate was selected for its biocompatibility [195], shear-thinning properties [196] and for its gelation ability in the presence of divalent cations [197]. Therefore, alginate acted as a gel strength enhancer, while it was desirable to maintain low NFC fiber content for optimal 3D cell culture **(I)**. Indeed, the addition of alginate improved the gel strength and viscosity significantly, when compared to the native NFC (**III**, Figure 1-2). For the NFCA threads and suture coatings, non-toxic crosslinkers Ca^{2+} and Ba^{2+} were used to stabilize the hydrogel structure. SK-HEP-1 and HepG2 cells were used as a co-culture in the NFCA hydrogels (Figure 8).

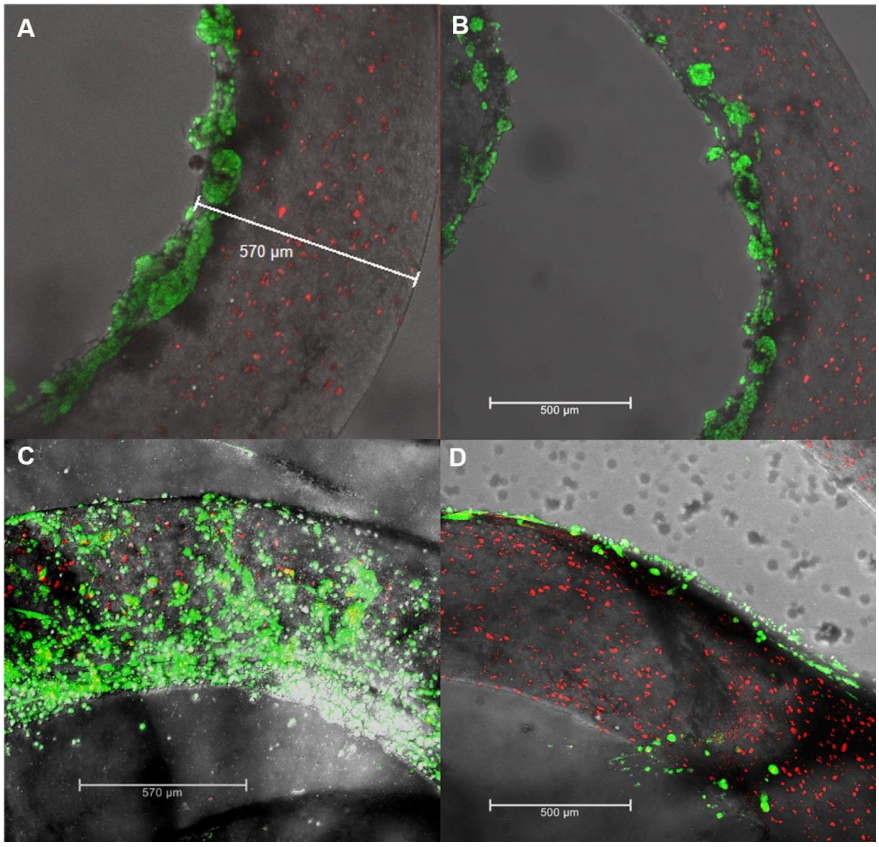

Figure 8. *HepG2 cell cultures and co-cultures of HepG2 and endothelial SK-HEP-1 cells. A-B) Spheroid forming HepG2 cells growing on the surface (green) and encapsulated within the NFCA matric (red). C-D) SK-HEP-1 cells monolayer formation on the surface (green) and HepG2 cells within the matrix (red). Typical morphologies of their respective cell types were observed.*

Surgical suture coatings were prepared with NFCA to cover a synthetic biogradable surgical suture. HepG2 cells were encapsulated within the NFCA matrix and sewn through pig liver segments to demonstrate cell delivery potential (Figure 9 A-B). The sutures coated with NFCA were used in an *ex vivo* study to investigate suturing performance (Figure 9 C-E). Various soft tissue of BALB/C mice and a Wistar rat were sutured and completed with knots. 12 out of 14 attempts were performed succesfully. The failure of suturing was indicated by the peeling off of the coating (**III**, Figure 6D). Peeling off was probably due to the hydrophobic nature of the surgical suture.

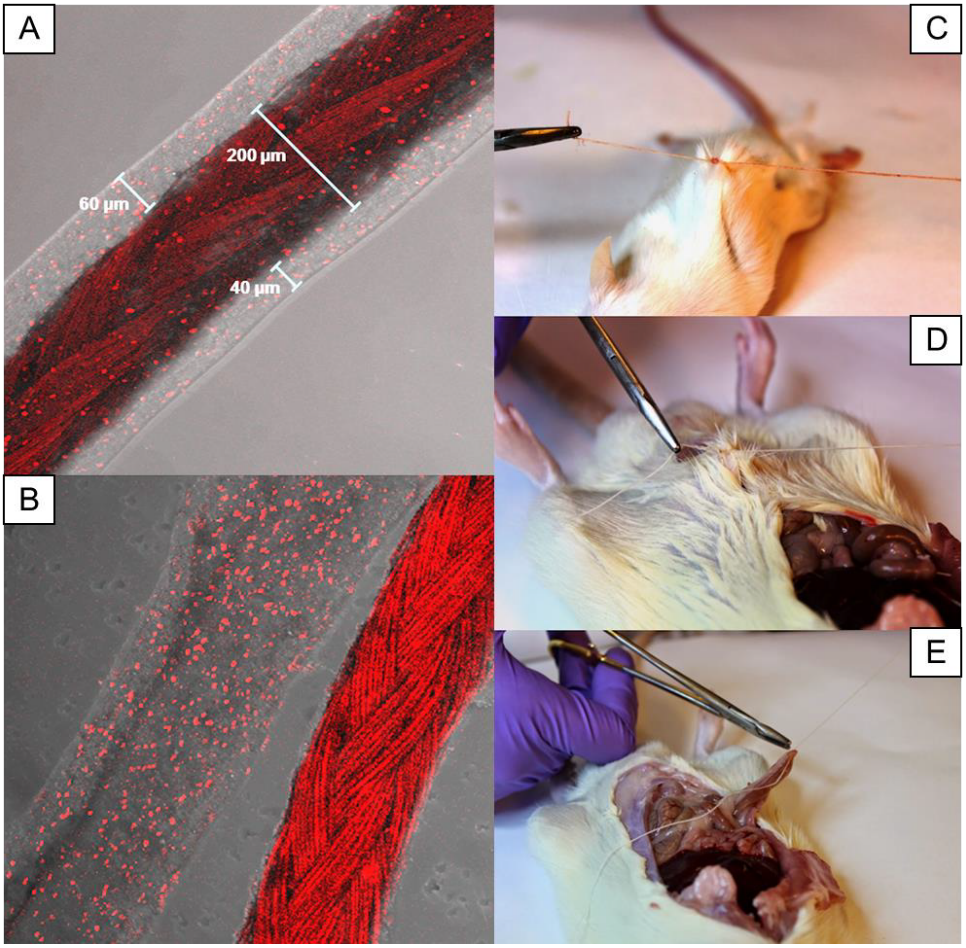

Figure 9. *NFCA coated surgical suture performance tests. HepG2 cells (red) encapsulated within the NFCA coating and sewn 3 times through pig liver segments shows A) intact coating structure, and B) coating removed from the suture manually. C-E) Various soft tissue sutures were made with NFCA coated sutures. 12 out of 14 attempts were successful.*

The NFCA encapsulated cell cultures remained viable during the whole 2-week study period (**III**, Figure 3), which was expected based on the excellent cell culturing abilities of NFC with low fiber content **(I)**. The addition of alginate did not decrease the cell culturing properties of the NFCA system. However, it enabled the formation of stronger gels while still retaining shear-thinning behavior. Shear-thinning was important in the fabrication of the suture coatings, where the high shear forces assist in the spreading of the NFCA evenly on top of the suture. NFC self-responsive gelation was responsible for keeping the structure intact during fabrication, while divalent cation cross-linking enabled the use of coated sutures. Cross-linking was used to stabilize the coating to withstand the handling and suturing processes of the intended application. However, some peeling off was still observed, which was designated as a suturing failure in 2 out of 14 attempts. The durability of the coating could potentially be improved with further optimization of the fabrication method and with the use of less hydrophobic sutures.

NFCA coated sutures were easily fabricated and used in the suturing process. The sutures bent normally, allowing the completion of the sutures with surgical knots. The advantages of this application are that the cells remain protected against host responses, it prevents cell distribution into unwanted areas and it enables a direct control over the number of cells, which are delivered. The amounts required for successful therapeutic delivery and effect is well within the capabilities of NFCA systems, as the number of cells used was similar when compared with other studies in cell therapy [198,199]. Additionally, the number of cells can be controlled within the NFCA by regulating the hydrogel coating thickness and cell seeding density, without the need to adjust suture length. Therefore, as suggested previously **(II)**, NFC-based hydrogels have excellent potential in cell delivery into easily accessible area, such as the skin. Additional potential target applications are diseases in the GI-tract, such as the Crohn's disease, where surgery is often required and cell therapy has been investigated as a treatment method [200-202]. Therefore, NFCA coatings could prove effective in combining the surgery and cell therapy into one treatment method.

NFC-based biomaterials for controlled drug delivery (IV)

The controlled release properties of high fiber content ANFC and low fiber content cross-linked ANFC were evaluated. Additionally, it was investigated if material handling processes, such as freeze-drying, impact on the drug release and rheological properties of the hydrogels. Furthermore, it was investigated if the use of cryoprotectants (PEG6000 and trehalose) are able to assist the preservation of original hydrogel structure upon rehydration of the aerogel. High fiber content ANFC viscosity (Figure 10) and storage/loss modulus (**IV**, Figure 3) changed upon freeze-drying and rehydration, probably due to lamellar aggregate formation through irreversible hydrogen bonding between the nanofibrils [203]. Therefore, the water used in the rehydration process is unable to completely rehydrate the aggregates. However, when cryoprotectants were included in the hydrogel mixture, the changes were negligible, as indicated by a complete restoration of its viscoelastic properties upon rehydration from the aerogel state. The preservation of these properties, especially during freeze-drying, has been a challenge for NFC-based materials [204,205]. The success in the new studies can be attributed to the cryoprotectants ability to limit aggregate formation of cellulose, therefore preserving the structural integrity of ANFC aerogels and enabling a more complete rehydration. This is important especially in pharmaceutical applications where the preservation of functional properties of the formulation (e.g. drug release properties) during the manufacturing processes is critical.

The addition of model compounds BSA (1 % wt/wt) and MZ (2 % wt/wt) affected the rehydrated rheological properties slightly (Figure 10). However, the addition of other compounds NAD (1.7 % wt/wt) and KETO (3.4 % wt/wt) had no discernible effect, which indicates that ANFC hydrogel rehydration is relatively insensitive of included drug compounds in the hydrogel mixture even at high concentraitons. Therefore, ANFC hydrogels can be loaded with excessive amounts of drug molecules with varying properties, which is important in utilizing the matrix structure of ANFC hydrogels in controlled drug delivery.

Low fiber content (1.1%) ANFC hydrogels cross-linked with crosslinking cations Al^{3+} (2.5 mmol/kg), Ca^{2+} (4.4 mmol/kg) and Fe^{3+} (2.2 mmol/kg) showed a significant increase in viscosity and storage modulus values (**IV**, Supplementary Figure S5). Loss modulus values were impacted to a lesser degree. However, the cross-linking stabilized to ANFC structure to withstand gel breaking at the tested frequencies (i.e. 1.1 % ANFC did not exhibit frequency independecy before cross-linking as indicated by the sudden change in loss modulus values at higher frequencies). Additionally, depending on the crosslinking cation, higher or lower values could be obtained. For example, the highest viscosity, storage and loss moduli were shown with Al^{3+}. Fe^{3+} and Ca^{2+} had a relavitely similar effects with each other, with Fe^{3+} having only a slightly greater impact on ANFC viscosity and loss modulus. This suggests that the viscoelastic properties of ANFC can be greatly affected without increasing the polymer fiber content, which has been indicated as a challenge for natural polymers [123]. Additionally, the preservation of hydrogel structure at high shear forces ensures that the viscoelastic properties remain after material handling processes, for example in coating applications **(III)**.

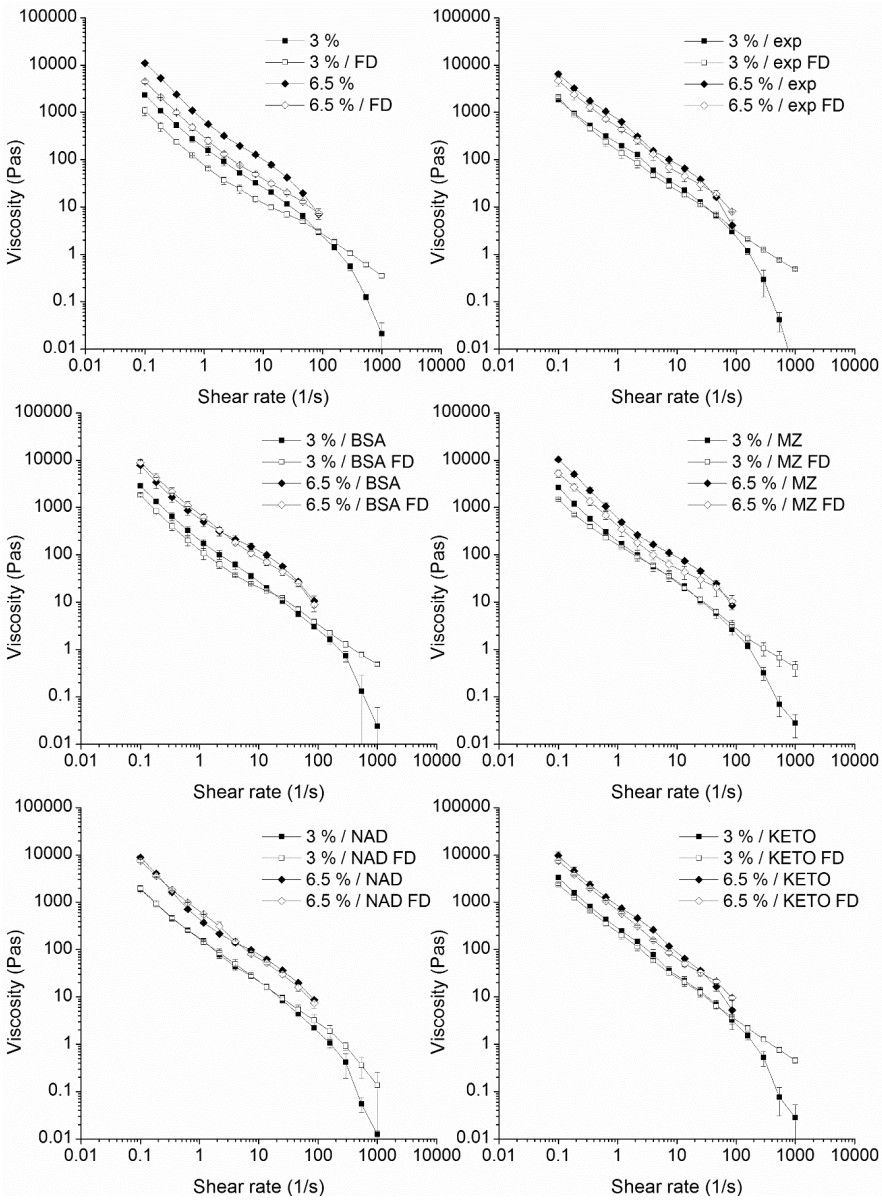

Figure 10. *3 % ANFC and 6.5 % ANFC shear rate viscosities. The effect of freeze-drying altered ANFC viscosities when rehydrated from the aerogel form (FD). The inclusion of cryoprotectants assisted rehydration and the viscosity values remain similar with the original hydrogel (exp). The addition of model compounds (BSA, MZ, NAD and KETO) had a slight or no effect on the rehydrated hydrogel viscosities.*

The freeze-drying and rehydration did not affect the drug release properties of ANFC hydrogels (Figure 11). It was observed that the ANFC hydrogels did not swell when submerged in the buffer during the drug release experiments, therefore the release of mechanism could be described according to the Fick's diffusion [206]. Additionally, while the hydrogel structure is important in controlling the release rate of drug compounds, the release of small molecules remain relatively unaffected by the changes in fiber content, as the amount of water bound within the system is still very high. The release of larger molecules can be somewhat more effectively controlled as it was shown with BSA and LZ (**IV**, Figure 4 A, C) and agrees with earlier discussion **(I)**.

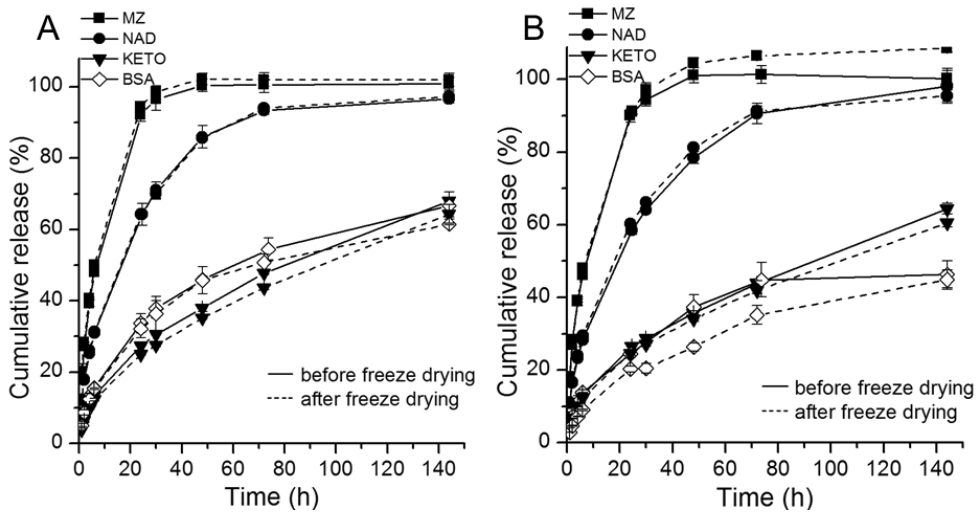

Figure 11. *Drug release properties of ANFC hydrogels before and after freeze-drying. A) 3 % ANFC hydrogels with drug compounds. B) 6.5 % ANFC hydrogels with drug compounds. No significant differences in the drug release properties were observed when aerogels were rehydrated.*

The effect of rehydration (Figure 11) and cross-linking (**IV**, Supplementary Figure S6) did not have any significant effect on the drug release properties of ANFC. With the use of cryoprotectants, ANFC hydrogels could be utilized in biomedical applications, which require freeze-drying processes, such as in preserving biological material, or simply to improve the shelf-life of sensitive compounds by keeping them in a dry state. It is important that the rheological characteristics can be completely restored, while the functionality remains unaffected. For example, cell culturing materials **(I)** or injectable *in situ* forming drug releasing implants **(II)** could be manufactured and stored safely until use, which would improve both shelf-life and logistics. This study further improves the understanding of the versatility of NFC-based materials, which could help to overcome previously daunting challenges related to the use of natural polymers.

NFC-based application in bioadhesion (V)

Bioadhesion is an important property of biological membranes. This property can be exploited in applications where adhesion to tissue or mucosa is preferable. This study focused on improving the bioadhesion properties of NFC, by incorporating mucoadhesive components within the matrix and therefore enabling the fabrication of bioadhesive films (Figure 12). Typically, bioadhesive applications are utilized to improve bioavailability by administering the application directly at the target site (e.g. wound healing) or by using alternative routes to avoid first-pass metabolism, such as a subcutaneous implant **(II)**. These systems can also benefit from controlled delivery applications **(IV)**.

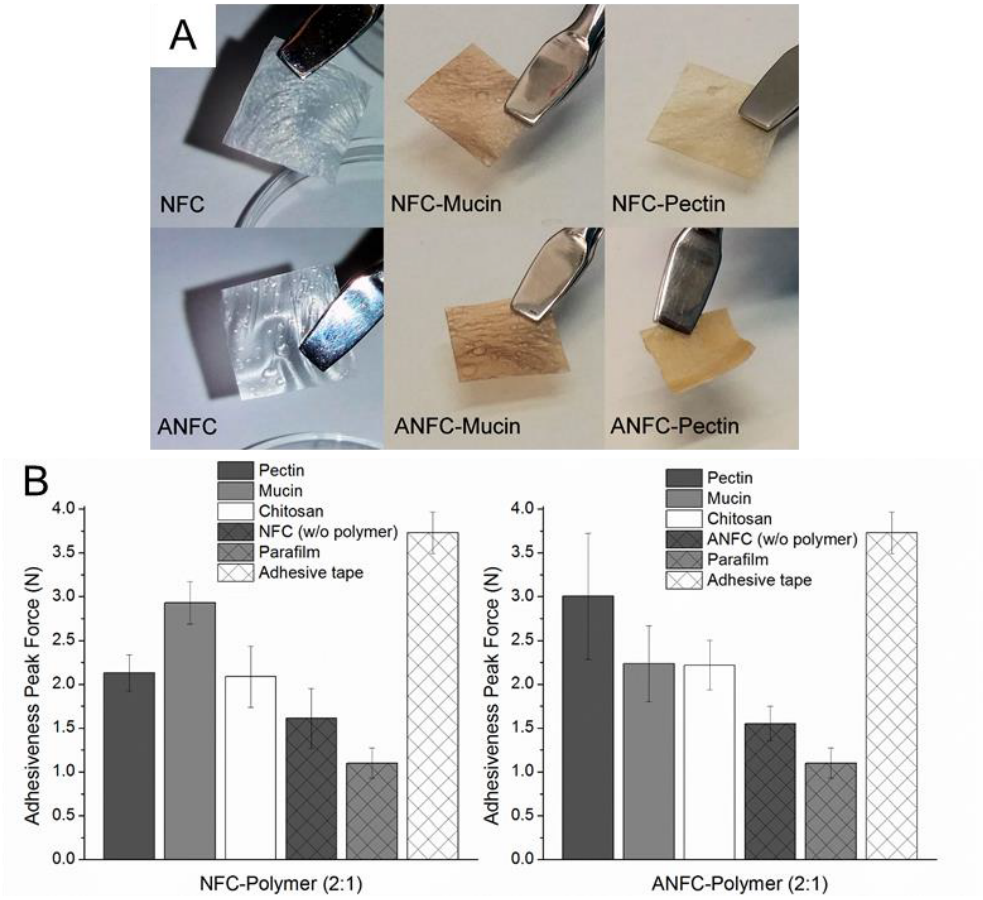

Figure 12. *Bioadhesion properties of NFC-based films. A) NFC-based films were fabricated, and B) tested for their adhesiveness. NFC-mucin and ANFC-pectin were the most adhesive combinations. Other combinations were also shown to exhibit increased bioadhesivity when compared to pure NFC/ANFC and parafilm as the negative control films.*

The adhesiveness of NFC films was greatly enhanced with the addition of mucoadhesive components (pectin, mucin or chitosan). Most adhesive combinations were NFC-mucin and ANFC-pectin. It is known that pectin can interact with cellulose, especially with the hemicelluloses present in the native NFC [207]. Therefore, all groups available for hydrogen bonding in the mucoadhesion process are not available and instead are spent in NFC-pectin interactions. ANFC hydrogels lack the hemicellulose components; therefore pectin-cellulose interactions are limited, resulting in enhanced mucoadhesion. As a cationic polymer, chitosan probably acts in a similar fashion, limiting the available groups for hydrogen bonding, relying more on polymer entanglement in the adhesion process.

The cytotoxicity and functionality of NFC-based bioadhesive films were also investigated in terms of drug release (Figure 13). No cytotoxicity was observed in any polymer combination. MZ containing films did induce some cytotoxicity, which agrees with previous literature about the toxicity of the drug itself [208].

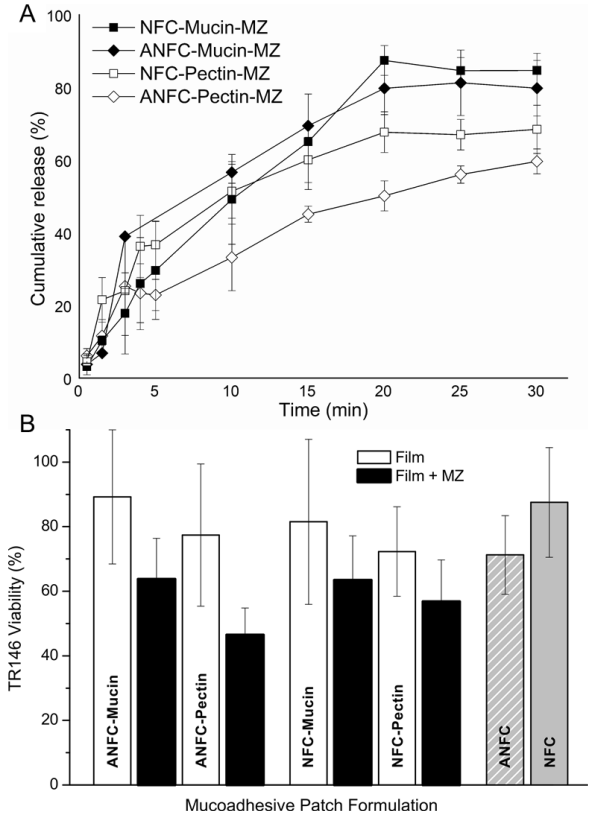

Figure 13. *Drug release properties and cytotoxicity of NFC-based bioadhesive films. A) Depending on the formulation 60-85 % of total amount was released before 30 min. B) Film formulation affect cytotoxicity. Films containing MZ were observed as slightly cytotoxic.*

Drug release was observed to be fast. All formulations expressed a burst of 20-40 % at the beginning of the experiment. This is probably due to MZ crystals dissolving at the surface of the films and therefore causing immediate release. The differences between individual formulations can be explained with morphological differences, such as film porosity observed in SEM migrographs (**IV**, Figure 3-4). The denser lamellar structure apparent in ANFC containing films could explain the slight differences in the released amounts. Additionally, films containing pectin swelled considerably more when compared to mucin films (**IV**, Table 4). The longer diffusion path most likely resulted in the slower release of MZ in pectin formulations.

Overall, NFC-based bioadhesive films were found to be highly adhesive. The films were biocompatible when evaluated for cytotoxicity in a TR146 buccal model cell culture. The functionality investigated in this study was drug release, which was observed as rapid. The potential application for such formulations could be the treatment of periodontal diseases. The films are administered on the surface of the oral cavity. Afterwards the film can be removed or swallowed, allowing the natural cleansing mechanisms to dispose of the film through the GI-tract. Formulation options could enable the controlled release of various drug compounds **(IV)**, or alternatively the mucoadhesive polymer combinations could be implemented to improve other NFC-based applications, where it is desirable that the system remains firmly at the target location **(II, III)**.

8 Future prospects

The need for functional biomaterials is growing more each year, as the understanding behind pathogenesis and disease states increases with new technologies, such as humanized animal models and microfluidics, that are able to study these topics in a physiologically relevant human-like environment. This provides more insight for biomaterial design, but also sets pressure on the development of more rational and effective biomedical applications. Many of these aspects can be handled with the design of next generation hydrogels that incorporate the potential of two or more polymer components within the same system. However, many currently used hydrogels are limited by availability, are animal-based, or require additional processing steps to achieve a functional state. Therefore, a suitable base material with all the necessary properties would further biomaterials research, if utilized rationally in the biomaterial design. Additionally, as the industry grows, more attention is shifted towards environmentally friendly "green" hydrogels and biomaterials that come from a sustainable source of safe materials free of ecological burden.

NFC is one of these "green" ecological materials, as it is derived from a nigh inexhaustible source (i.e. plants) and degrades naturally in the environment. Furthermore, as discussed and shown through experimentation in this thesis, many features of NFC can be exploited in designing hydrogel-based biomaterials and biomedical applications. NFC offers a base ingredient for the next generation hydrogels, as it possesses intrinsic properties that could prove advantageous in biomaterial design in the future. The ideal situation includes an easily available source of a biocompatible and biodegradable material, which can be sculpted into shape and tuned with high precision as the application demands. So far, NFC fits in most of these categories, with the exception of biodegradability *in vivo*. Therefore, there are aspects which still limit the use of NFC and require further investigation. Fortunately, NFC provides possibilities through chemical modifications, which may yet lead to a discovery of *in vivo* self-degrading NFC. Additionally, with the advances in 3D bio-printing technologies, further options become available in structural design, as NFC can serve as a bioink.

Plant-derived NFC is currently underrepresented in biomedical application research when compared to bacterial cellulose. However, as new chemical modifications and combinations of next generation hydrogels are discovered, new functional properties can be implemented and utilized. Therefore, future research in new functional biomedical applications is promising for the use of plant-derived NFC, and NFC-based biomaterials. As they offer a potential way to bridge the gap between *in vitro* and *in vivo*, leading to a future of safe and efficient, green biomaterials.

9 Conclusions

Plant-derived nanofibrillar cellulose (NFC) is a versatile biomaterial with various potential uses as a biomedical application. NFC possesses intrinsic physical and mechanical properties that resemble *in vivo*-like microenvironment, which can be further tuned and modified with the addition of other polymers, cross-linking agents or by adjusting the fiber content to match the properties of the target soft tissue. Most notably, high water content, material stiffness and viscoelastic properties offer an excellent platform for cell culture, therefore providing ideal conditions for achieving a natural cell morphology, functionality and preservation of the desired phenotype **(I)**. Additionally, NFC is a pseudoplastic material, which enables injectability through a reversible shear-thinning behavior. This allows material delivery with minimal invasive procedures, such as an implant for drug or cell delivery **(II)**, and it enables fabrication processes where shear-thinning is advantageous, such as coating applications **(III)**. The self-gelation occurs spontaneously, therefore additional cross-linkers or external stimuli are not necessary. However, the chemical modification properties enable many additional features, such as controlled drug delivery **(IV)**, functionalization, e.g. bioadhesion **(V)**, and fine tuning rheological properties without adjusting fiber content. Therefore, plant-derived NFC provides great potential for future biomedical applications, due to its biomimetic properties and outstanding versatility.

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